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(54) Title: PEPTIDE ANALOGS OF THE FACTOR IXa PLATELET BINDING SITE

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PEPTIDE ANALOGS OF THE FACTOR IXa PLATELET BINDING SITE

Field of the Invention

The invention relates to synthetic peptide analogs of the factor IXa platelet binding site.

Background of the Invention

Factor IXa.

Human factor IXa is the activated form of the zymogen, factor IX. The non-activated form of factor IX is a single-chain glycoprotein of 415 residues and has considerable amino acid sequence similarities with other vitamin K-dependent proteins, such as factor VII, factor X, prothrombin, and protein C. Kurachi, et al. Proc. Natl. Acad. Sci. USA 79 6461-6464 (1982) (incorporated herein by reference) disclose the amino acid sequence of factor IX, deduced from the sequence of a cDNA insert coding for factor IX. The most easily definable functional domains of the factor IX sequence (as described from the amino to the carboxyl terminus, respectively) are: the vitamin K-dependent domain containing post-translationally modified glutamic acid residues (γ-carboxyglutamic acid or "Gla"; two epidermal growth factor (EGF) - like domain residues (which include an endothelial cell binding site); an activation peptide region; and a catalytic domain, which confers the protease function.

Factor IX activation to factor IXa involves a two-step mechanism. The Arg^{145} - Ala^{146} bond is cleaved, giving rise to a two-chain intermediate, which is then cleaved at the Arg^{180} - Val^{181} bond. The second cleavage yields factor IXa as a disulfide-linked serine protease and a 35-residue activation peptide having a molecular weight of 11 kDa. During hemostasis, factor IX may be activated by factor XIa in the presence of Ca^{2+} ions or by factor VIIa in a reaction

requiring Ca^{2+} ions and tissue factor. Both enzymes cleave the Arg^{145} - Ala^{146} and Arg^{180} - Val^{181} bonds in factor IX.

The activated form of factor IX, factor IXa, is a disulfide-linked serine protease which forms a complex with its cofactor, factor VIIIa. Factor IXa and factor VIIIa bind in close proximity to one another on the platelet surface, which facilitates the formation of the complex. Factor IXa catalyzes the activation of factor X to factor Xa by hydrolyzing internal arginine-isoleucine and arginine-glycine bonds in the factor X heavy chain.

The hydrolysis of factor X by factor IXa provides two active molecules of factor Xa. Each factor Xa molecule can associate with factor Va and calcium ions to form a prothrombinase complex on a negatively charged phospholipid surface (e.g., a platelet surface). The resulting prothrombinase complex then converts prothrombin to thrombin, which in turn catalyzes the conversion of fibrinogen to fibrin and results in clot formation. Thus, platelet binding of factor IXa and conversion of factor X to factor Xa by the platelet-bound factor IXa/factor VIII complex are essential steps for efficient clot formation in the intrinsic blood-clotting cascade.

Both human factor IX and factor IXa compete with one another to bind reversibly to 250-300 shared binding sites on the surface membrane of an activated platelet. Additionally, factor IXa binds to 250-300 more sites to which factor IX cannot bind. Hence, factor IXa can bind to twice the number of platelet sites (500-600 per platelet) as factor IX (250-300 sites per platelet). Furthermore, the presence of saturating concentrations of factor VIIIa and factor X can increase the affinity of factor IXa binding five fold (from a K_d (dissociation constant) of approximately 2.5 nmol\L to about 0.5 nmol\L). But, the presence of factor VIIIa and factor X does not affect the affinity of factor IX binding (K_d of approximately 2.5 nmol\L). Thus, the presence of factors VIIIa and X favor the binding of factor IXa over factor IX.

Moreover, platelet binding site occupancy with factor IXa is closely coupled with rate enhancements of factor

X activation to factor Xa. These rate enhancements are achieved as a consequence of a decrease in K_m (Michaelis constant) by activated platelets to a value near the plasma concentration of factor X, combined with an increase in K_{cat} (turnover number, i.e. amount of inactive factor X enzymatically converted to its active form) in the presence of activated platelets and factor VIIIa.

The roles of Ca^{2+} concentration, the catalytic active site residues, and the Gla domain residues inbinding factor IXa on the activated platelet surface and in the assembly of the factor X activating complex have been studied. Only full-length factor IX or modified full-length factor IX was utilized for the above studies. In Factor IX, either an average of 1-3 γ-carboxyglutamic acid residues were chemically modified (Mod-Gla) or all γ-carboxyglutamic acid residues were enzymatically removed (Des-Gla). See, Rawala-Sheikh et al., Blood 79 398-405 (1992).

The enzymatic active site of factor IXa, which is specific for factor X, may be blocked by reaction with a peptide such as glutamyl-glycyl-arginyl-chloromethyl-ketone. Such full-length active site-inhibited factor IXa molecules are competitive inhibitors of both factor IXa binding to platelets and factor X activation. The K_i (inhibition constant) for factor IXa with its enzymatic active site-blocked is identical to the K_d for factor IXa binding to platelets. See, Ahmad et al. J. Biol. Chem. 264 20012-20016 (1989). Thus, the catalytic active site of factor IXa is not involved in factor IXa binding to platelet receptors. However, the modification of as little as one mole of Gla per mole of factor IX results in a complete loss of factor IXa-related coagulant activity even though the factor IXa Gla domain is not the active enzymatic site. See Rawala-Sheikh, et al. Blood, 79 398-405 (1992) at page 402. This loss of activity apparently occurs when any one of eleven of the twelve Gla residues of factor IX is modified.

The factor IX Gla domain has been studied because of its relationship to the binding of factor IXa to endothelial cells. Native peptide fragments comprising the intact Gla domain of factor IX were prepared by partial digestion

of factor IX with enzymes. The partial digestion provided native peptide fragments predominantly comprising at least factor IX amino acid residues 1-42 which were purified by eluting through a Sephadex column. These native fragments of factor IX comprising the intact Gla domain inhibited the binding of factor IX to endothelium, see Ryan et al. J. Biol. Chem 264 20283-20287 (1989).

Further, a computer-generated model approximating the human factor IX Gla sequence which contains a binding site for endothelial cells was provided by Cheung, et al. J. Biol. Chem. 267 20529-20531 (1992). The computer-generated model was produced as a hypothetical approximation based on coordinates from the bovine calcium prothrombin crystalline structure. A model was produced which theoretically implicated amino acid residues 3-11 as a binding site to endothelial cells. The title of the Cheung, et al. article states that the endothelial binding site is made up of amino acid residues 3-11. However, at page 20531, col. 1, in vitro testing is described which showed that atom 11 was not part of the endothelial cell binding site. Bovine and human factor IX were found to bind equally well to bovine endothelial cells even though human factor IX has glutamine and bovine factor IX has arginine at position 11. Thus, in contrast with the hypothetical model of Cheung, et al. the in vitro tests of Cheung, et al. indicate that amino acid position number 11 of the Gla domain is not part of the factor IX endothelial cell binding site.

The accuracy of the computer model of Cheung, et al., supra, is questionable, since it does not explain the in vitro test results. Perhaps, the inconsistencies between the in vitro test data and the proposed theoretical model of the endothelial cell binding site of factor IX results from the model being an approximation. The Cheung model is hypothetical and unverified since it is based on prothrombin crystalline data and approximations from a computer program to provide a theoretical three-dimensional model of the factor IX Gla domain. Moreover, the coordinates of the Cheung, et al, computer model are not listed. Thus, the model cannot be generated. Cheung et al. synthesized DNA constructs for

producing mutant full-length factor IX by recombinant DNA techniques. Only full-length factor IX molecules were produced. The synthetic DNA constructs coded for mutant factor IX molecules containing Gla domain modifications in amino acids 1-11. All of the mutant factor IX molecules produced, preserved the two adjacent γ-carboxyglutamic acid (Gla) amino acids at positions 7 and 8 in the Gla domain. Only amino acids other than Gla amino acids were varied. Preservation of the Gla-7 and Gla-8 was necessary, since the Gla amino acids located in the Gla domain of factor IX associate with calcium ions which are critical to the three-dimensional constraint of the Gla domain in the intact factor IX. There were no attempts by Cheung, et al., supra, to synthesize short peptides of Gla domain amino acid residues as candidates for inhibiting the binding of human factor IX to endothelium cells.

The Gla-domain of factor IXa has also been implicated as being involved in the binding of factor IX to a platelet receptor. See Rawala-Sheikh, et al. Blood 79 398-405 (1992). Mutant full-length factor IXa produced by recombinant DNA techniques has been studied to investigate the role of Gla residues in the binding of factor IXa to the platelet surface. Tests with these mutant factor IXa molecules indicated that other determinants outside of the Gla domain were also responsible for the binding of factor IXa. See Ahmad et al. J. Biol. Chem. 268 8571-8576 (1992). Two of the factor IX mutants studied are chimeric proteins of factor IX wherein the EGF-1 and EGF-2 amino acid sequence segments of factor X have been substituted for the N-terminal EGF-1 of factor IX (IX_{xegf1}) or the C-terminal EGF-2 domain of factor IX (IX_{xegf2}), respectively. The studies with intact chimeric activated factor IX_{xegf1} (factor IXa_{xegf1}) suggest either (1) that the EGF-1 domain of factor IX/IXa is not involved in factor IX/IXa binding to platelets, or (2) that the EGF-1 domain from factor X, when inserted into factor IX, suffices to promote normal factor IX/IXa binding.

In nature the Gla amino acid residues of the factor IX Gla domain are synthesized as glutamic acid residues which are post-translationally modified to the dicarboxylic Gla form

by a vitamin-K dependent carboxylase. Thus, it is difficult to synthesize peptides comprising the critical γ-carboxy-glutamic acid residues. Hence, recombinant DNA techniques have been used previously, which provide mutant Gla domains located in full-length factor IXa. These techniques take advantage of post-translational modification of glutamic acid to Gla by host enzymes.

Synthetic peptide analogs of the factor IXa binding site, which do not contain the Gla amino acid residues at amino acid positions 7 and 8 of the Gla domain, have not been produced prior to the present invention. Moreover, it was not known that short synthetically conformationally restricted peptides free of Gla amino acid residues that are adjacent to one another could compete with factor IXa for binding sites on the activated platelet surface.

Antithrombotic Therapy.

Existing methods for preventing or treating arterial and venous thrombosis involve inhibiting both the intrinsic and extrinsic blood coagulation cascades with oral anticoagulants, heparin or other anticoagulants, or alternatively by pharmacologically inhibiting platelets. For example, oral anticoagulants such as coumarin-like drugs are used to inhibit the syntheses of vitamin K-dependent proteins. They block many coagulation reactions, involving proteins such as prothrombin, factor VII, factor IX and factor X. Heparin, by potentiating the action of antithrombin III, accelerates inactivation of thrombin, factor Xa and a variety of other plasma serine proteases.

These therapeutic approaches are nonselective and inhibit coagulation reactions involved in the development of venous and arterial thrombosis while at the same time inhibiting reactions which are essential for the maintenance of normal hemostasis. Similarly, most platelet inhibitor drugs block a wide variety of platelet responses. Thus, while some drugs may be effective in preventing thrombotic processes, they can enhance the risk of bleeding. What is needed is a therapeutic agent which specifically interferes with intrinsic coagulation reactions leading to the activation of factor X,

while leaving extrinsic coagulation reactions intact. This will permit normal hemostatic plug formation at sites of vascular injury (extrinsic coagulation), thereby minimizing the risk of bleeding during the antithrombotic therapy.

Prevention of factor IXa binding to activated platelets would limit the biologically important platelet contribution to intrinsic coagulation reactions. Accordingly, effective anti-thrombotic agents which inhibit the binding of factor IXa to surfaces of activated platelets are needed.

Summary of the Invention

A synthetic peptide is provided having an amino acid sequence length of from at least 5 to about 75 amino-acids, which sequence comprises an amino acid sequence segment corresponding to a portion of the sequence of the binding site on factor IXa for activated platelets, or a pharmaceutically acceptable salt of the peptide;

wherein the peptide has an artificially restricted conformation and is free of adjacent γ-carboxyglutamic acid residues; and

wherein the peptide has the ability to inhibit the binding of factor IXa to an activated platelet surface. Preferably, the synthetic peptide is free of γ-carboxyglutamic acid residues.

In another embodiment, a synthetic peptide is provided having an amino acid sequence length of from at least 5 to about 75 amino acids, which sequence comprises an amino acid sequence segment corresponding to a portion of the sequence of the binding site on factor IXa for activated platelets, or a pharmaceutically acceptable salt of the peptide;

wherein the peptide has an artificially restricted conformation provided at least in part by a covalent bond other than a cysteine-cysteine disulfide bond, or by a cysteine-cysteine disulfide bond which comprises at least one cysteine residue not present in the corresponding native factor IXa amino acid sequence; and

wherein the peptide has the ability to inhibit the binding of factor IXa to an activated platelet surface.

In a further embodiment the invention is directed to a method of designing and synthesizing a synthetic peptide analog to the site on the factor IXa chain for binding to activated platelets. The distance between two parts of a molecular model of the factor IXa platelet binding site is determined at conformational equilibrium. The primary structure of the binding site is then modified to restrict that distance to the determined distance. The designed peptide analog having from at least five to about seventy-five amino acids and comprising the modified primary structure is then synthesized.

The invention further provides pharmaceutical compositions comprising one or more of the peptides according to the invention corresponding to the portion of the sequence of the binding site for activated platelets on the factor IXa chain, or a pharmaceutically acceptable salt thereof, in combination with a pharmaceutically acceptable carrier. Preferred pharmaceutical compositions comprise a peptide according to the invention having an amino acid sequence from five to about forty-five amino acids in length, and more preferred compositions comprise a peptide having an amino acid sequence from five to about twenty amino acids in length.

The invention also provides a method of inhibiting the binding of factor IXa to the surface of an activated platelet. The activated platelets are contacted with one or more peptides of the invention, corresponding to a portion of the sequence of the binding site for activated platelets on the factor IXa chain, which compete with factor IXa in binding to the activated platelet. Inhibition of factor IXa attachment to the platelet surface, and factor IXa enzymatic activity on the platelet surface, inhibits factor IXa coagulant activity. Thus, the peptides of the invention are potent anticoagulants, having antithrombotic utility.

By "platelet binding sites" or "activated platelet binding site" on factor IX or factor IXa is meant the region of the intact IX mature polypeptide chain comprising from amino acid 1 (Tyr) to about amino acid 14 (Leu) of the mature

polypeptide, corresponding to amino acids 1-14 of SEQ ID NO:1.

By an amino acid sequence which "corresponds to a portion of the platelet binding site" on the factor IXa chain is meant a sequence which comprises a sequence segment identical to a portion of the platelet binding site sequence or a sequence segment derived from a three-dimensional model of a portion of the platelet binding site sequence.

By "sequence segment" it is meant a continuous portion of an amino acid sequence comprising two or more amino acid residues.

Detailed Description of the Invention

We prepared synthetic peptides based upon a testable computer model to approximating the platelet binding site of factor IX. The synthetic peptides, artificially constrained to the three-dimensional structure of our computer model (see Appendix 1 below), are potent inhibitors of factor IXa binding to platelets. The fact that the constrained, synthetic peptides are potent inhibitors of factor IXa binding to platelets establishes that our computer model is an accurate three-dimensional representation of the factor IXa platelet binding site.

The synthetic peptides compete with factor IXa for binding sites on the platelet surface. Activation of factor IX to factor IXa and the expression of factor IXa enzymatic activity on the platelet surface are key biological events in hemostasis. The binding of factor IXa to platelets is essential for efficient factor X activation. Prevention of factor IXa binding to activated platelets inhibits the biologically important platelet contribution to coagulation reactions involving factor IXa. Thus, the synthetic peptides provide a potent therapeutic effect by inhibiting the binding of factor IXa to platelets. Importantly, peptides which have the same amino acid sequence as the constrained peptides, but not constrained to the three-dimensional structure of the computer model, are inactive or substantially less active than the constrained peptides.

Computer modeling has provided a testable three-dimensional representation of the factor IXa platelet binding

site. The space-filling model was calculated by utilizing the calculated structure's primary amino acid sequence. Hypothetical disulfide linkages were located within the Gla domain model equal to the distances between molecules.

The computer model of the Gla domain three-dimensional structure was constructed by starting with the coordinates for the crystalline structure of the prothrombin fragment 1 Gla domain (Soriano-Garcia, et al. Biochem. 31 2554-2566, (1992). The initial coordinates were developed further by changing the model's prothrombin Gla domain amino acid sequence to the amino acid sequence of residues 1 to 47 of the factor IX Gla domain. The three-dimensional mathematical modeling was performed using the biopolymer module provided within the SYBYL computational chemistry package. The Amber force field, as implemented in SYBYL, was utilized in all the subsequent calculations. Atomic parameters describing calcium and the γ-carboxylated glutamic acid residues were added to the force field tables to increase the accuracy of the computer model.

After the changes to the model due to the amino acid replacements were completed, the structure was energy minimized to convergence using a conjugate-gradient approach. The newly minimized structure was then solvated with water using the Silverware algorithm as implemented in SYBYL. The water/protein complex was again energy minimized prior to an energy-dependent simulation of molecular motion.

Our resulting three-dimensional model of the Gla domain structure of factor IXa was used as a design template for synthesizing constrained peptides according to the present invention. These peptides are expected to adopt a conformational repertoire overlapping that of the native protein. The modified, constrained peptides identified herein are free of adjacent γ-carboxyglutamic acid residues except when they are constrained by a covalent bond other than a cysteine-cysteine disulfide bond. The synthetic peptides inhibit factor IXa binding to platelets. Thus the peptides according to the present invention are potent anticoagulates, which are believed useful as antithrombotic agents.

Ideally, an antithrombotic agent should interfere with intrinsic coagulation reactions leading to the activation of factors XI and IX while leaving extrinsic coagulation reactions intact. Normal hemostatic plug formation can occur at sites of vascular injury via intact extrinsic coagulation reactions. The synthetic peptides of the invention are specific for the platelet binding site on factor IXa and have the ability to inhibit the binding of factor IXa to platelets. Thus, the synthetic peptides will inhibit factor IXa enzymatic activity on the surface of platelets, without affecting the extrinsic pathway of blood coagulation involving factor VII, X and V, and prothrombin.

The inventive peptides are specific in their inhibitory effect on the intrinsic coagulation pathway. Hence, the peptides will inhibit or minimize intravascular thrombus formation without sacrificing normal hemostatic plug formation.

Traditional synthesis of the linear amino acid sequence of biologically interesting proteins may result in peptides that are either biologically inactive or, at best, marginally active. Previously the three-dimensional structure of the factor IX has been preserved by modifying the binding site portion of the molecule and leaving the rest of the molecule intact. For example, activity of the modified binding site of factor IX with endothelial cells depended upon the intact molecule to provide a three-dimensional conformation.

Our molecular model of the factor IXa Gla domain provides a template for designing conformationally-restricted synthetic analogs to the factor IXa site which binds to the platelet surface. The synthetic conformationally-restricted analogs have the ability to compete with factor IX and factor IXa for binding sites on platelet surface. Thus, the synthetic analogs inhibit the binding of factor IX or factor IXa to sites on the platelet surface. This in turn prevents the enzymatic activity of factor IXa from being expressed on the platelet surface.

Factor IXa binds to 500-600 binding sites per platelet. Factor IX will only bind to half of those sites.

Thus, the constrained peptides mimic factor IXa, since they have the ability to bind to all 500-600 platelet sites. Using both distance and geometric constraints imparted through measurement of the subdomains within the calculated factor IXa binding site structure, constraints are artificially introduced, e.g., disulfide bonds to limit the conformational freedom of a synthetic peptide that incorporates the relevant amino acids. Certain conformationally restricted synthetic analogs having the ability to inhibit the binding of factor IX and factor IXa to platelets correspond to factor IXa chain residues 3-11, according to the numbering of the amino acids of the mature polypeptide. The model disclosed may be utilized to prepare additional conformationally-restricted synthetic peptides having similar activity.

Appendix 1 included herein contains the set of Brookhaven coordinates and connect statements specifying our equilibrium conformation model of the major portion of factor IXa Gla chain domain comprising the 48 amino acids spanning positions Tyr 1 To Gly 48, inclusive. (SEQ ID NO:1, amino acids 1-48). The remaining amino acids of the factor IXa sequence were truncated.

A corresponding graphic molecular model satisfying these coordinates may be generated by inputting the coordinates and connect statements into any of the many commercially available molecular modeling programs which are capable of reading files in the Brookhaven format. Such programs include, for example, those of BioDesign, Inc., Pasadena, CA; Biosym Technologies, San Diego, CA; Tripos, St. Louis, MO; Polygen, Waltham, MA; and Chemical Design Ltd., Oxford, UK. The data may be entered as an ASCII file.

According to the Brookhaven format shown in Appendix 1, each of the atoms of factor IXa chain Gla domain, residues 1-48 is assigned a number and respective X, Y and Z coordinates. The coordinate portion of the listing begins with the Tyrosine residue (Tyr 1) at position one of the mature factor IXa heavy chain. The atom types are identified as "N" for nitrogen, "HN" for hydrogen which is connected to a nitrogen atom, "C" for carbon, "CA" for α carbon, "CB" for β carbon, "CG" for γ carbon, and so forth. Identical atoms

of branched side chains are indicated by numbers. Thus, the two γ carbons of VAL 10 are designated "CG 1" and "CG 2" respectively.

The data file further comprises a connect statement which begins immediately after the coordinates for atom 539. The connect statement identifies the covalent bonding pattern of each of the 525 atoms of the 48 amino acids residue chain and atoms 526-539 which are the unnamed carboxylic terminating sequence. The complete data file of 525 coordinates, together with the connect statement for these entries, specifies the equilibrium conformation of the factor IXa Gla domain.

The amino acid sequence in the computer model can be modified as follows to eliminate Gla amino acid residues in the three-dimensional structure and maintain the binding surfaces represented by amino acid Gly 4 - Lys 5 - Leu 6 and by Phe 9 - Val 10 - Gln 11. The Gla residues at position 7 and 8 are changed to asparginine and glutamine residues, respectively. Also proline amino acid residues are substituted for serine at position 3 and glycine at position 11. Surprisingly, these changes which eliminate the two Gla amino acid residues introduce a folding pattern similar to that present in the native structure. The predicted folding pattern for this putative structure was tested for its ability to mimic the structure observed in our model of the Gla domain of factor IXa. Satisfactory agreement was found between our proposed model according to Appendix 1 and the modified putative structure.

The analogs of the invention generally have an amino acid sequence similar to the native Gla domain sequence in the vicinity of the platelet binding site. However, a covalent modification is artificially introduced to restrict each analog to the conformation (or one close to it) displayed by the above model. Preferably, the analogs consist essentially of a peptide having from at least five (5) to about seventy-five (75) amino acid residues. Preferably the analog has at least five (5) to about forty-five (45) amino acid residues, most preferably from about five (5) to about twenty (20) amino acid residues. Generally, the covalent modification is accomplished by determining a distance

between two noncontiguous parts of the amino acid chain according to the model. Then a chemical moiety is introduced to fix that determined distance in the analog. For example, a 5-6Å distance can be fixed using a disulfide bond. Cysteine residues can be introduced at the appropriate positions in the model followed by testing the new cysteine-containing model for its ability to mimic the structure observed in the model. Alternatively, the disulfide bond can be artificially introduced by generating a disulfide bond between native cysteine residues in the synthetic polypeptide when this will produce a polypeptide with a restricted conformation corresponding to the above model.

In constraining the peptide analogs it is sometimes necessary to compensate for the orientation of amino acid side chains such that torsional stress does not misalign the peptide structure. Thus, in some instances, it is desirable to employ D-Cys analogs or appropriate combinations of D-L cysteine to mimic the correct stereochemistry. In general, these peptides are then synthesized according to the standard chemistry described below.

The use of native or artificially introduced cysteine residues to create the artificially introduced disulfide bridge is one way to conformationally restrict the peptides. Disulfide bonds, however, can be intrinsically unstable and it is sometimes difficult to obtain a homogeneous solution of intradisulfide-bonded species without concomitant mixed disulfides. If a biologically active conformationally restricted peptide having a cysteine-cysteine disulfide bond tends to unfold, it may be more effective to constrain the peptide in a folded conformation via a covalent bond which is more stable than a disulfide bridge. There are several strategies which can be utilized in the covalent closure of the peptides. Two of these strategies are described below.

The peptide can be internally cross-linked via the side chains of a lysine ϵ-amino group and the carboxylic acid function of a glutamic or aspartic acid side chain, thus creating an amide bond. The peptide is synthesized according to standard procedures on a low substitution (0.2 mmol/gm or less) paramethylbenzhydrylamine resin. The first residue

added to the resin is an N-α-tBOC,ε-fMOC lysine. The rest of the peptide synthesis is continued normally using tBOC chemistry until the final residue is added. The last residue to be added is a Z-protected glutamic acid, where the carboxylic acid moiety is protected with a tertbutyl group. Treatment of the peptide resin with piperidine/DMF removes the fMOC group from the ε-amino group of the initial lysine without affecting any other protection groups. Subsequent treatment with trifluoroacetic acid removes the protection of the carboxylic acid group of the glutamic acid. Following neutralization, the peptide is covalently closed using a standard diimide-mediated coupling reaction. It should be emphasized that this is only one of the ways in which the synthetic peptide can be covalently closed.

Other fMOC/tBOC strategies include covalent closure of the peptide between two free amino groups utilizing toluene-2,4-diisocyanate (TDI), a heterobifunctional cross-linker. The methyl group of the aromatic ring of TDI prevents the isocyanate group in the 2 position from reacting at a pH 7.5 or below, whereas the isocyanate group in the para position is highly reactive. A shift in pH to greater than 9.0 will initiate a reaction with the isocyanate group in the 2-position, thus enabling highly specific and controlled conditions for covalent closure of the peptide.

By utilizing a variety of different strategies for restricting the conformation of peptides, distance geometries and orientation of the folded peptide can be controlled. Any such strategies employing chemical reactions known in the art may be used.

Using the above described techniques, synthetic peptide analogs can be made and tested for their ability to inhibit factor IXa binding to the platelet surface and to inhibit factor IXa enzymatic activity on the platelet surface.

Particularly useful peptide analogs which were derived using the techniques described herein comprise amino acids corresponding to segments of the factor IXa Gla domain sequence residues 1-14. Conformationally restricted peptides corresponding to factor IXa residues 4-11 (SEQ ID NO: 2) and residues 4-8 and 9-11 (SEQ ID NOS: 3-4), respectively. The

predicted folding pattern of the peptides were tested for their ability to mimic the structure observed in our model of the Gla domain of factor IXa. Finding satisfactory agreement, the peptides were synthesized according to conventional solid phase procedures on an Applied Biosystems 430A Peptide Synthesizer by a modification of the procedure described by Kent et al. in Synthetic Peptides in Biology and Medicine eds. Alitalo et al. (Elsevier Science Publishers, Amsterdam, pp. 29-58 (1985)), in which dimethylformamide replaced methylene chloride in the routine wash cycles. The synthesis was carried out using a paramethylbenzhydrylamine resin (United States Biochemical Corp., Cleveland, OH). The solvents and protected amino acids were synthesis grade biotechnology products purchased from Fischer Scientific Co., Pittsburgh, PA. The resulting peptide was folded into a three-dimensional constrained conformation in a separate chemical reaction step after the peptide was purified.

The folded peptides were examined by both reverse phase and gel filtration high performance liquid chromatography (HPLC). Each of the three folded peptides demonstrated a single homogenous peak with a retention time identical to the corresponding unfolded peptide. This indicated the presence of a single homogeneous mixture for each folded peptide, and not a mixed population of diverse polymers.

The hexapeptide, SEQ ID NO:3, has an amino acid sequence identical to Factor IXa amino acids 2-7, except that the first and last amino acids have been replaced with cysteine residues. Similarly the peptide, SEQ ID NO:4, is identical to factor IXa amino acids 8-12, except for the replacement of the first and last amino acids with cysteine residues.

The peptide, SEQ ID NO:2, is identical to amino acids 2-13 of native factor IXa except (i) the first and last amino acids (Asn 2 and Asn 13) are replaced with cysteine residues; (ii) the serine corresponding to position 3 and the glycine corresponding to position 12 of the native peptide are replaced with proline residues; and (iii) the two Gla residues at position 7 and 8 are replaced with Asp and Glu residues, respectively. Hence, the SEQ ID NO:2 peptide

consists of the amino acid sequence Cys - Pro - Gly - Lys - Leu - Asp - Glu - Phe - Val - Gln - Pro - Cys.

Each of the three peptides, SEQ ID NOS: 2-4, were conformationally restricted using cysteine-cysteine disulfide bonds. Other restricting means may be advantageously used. Each peptide inhibits the binding of factor IXa to the platelet surface. As a consequence, each peptide may be used to inhibit the procoagulant function of factor IXa by limiting platelet involvement in intrinsic coagulation. Methods of assaying factor IXa binding to the platelet surface are known in the art. One such method is described hereinafter in Example 5(d).

The present peptides are relatively short in length and therefore they are easily synthesized by chemical means. Moreover, the peptides are preferably free of the traditionally difficult-to-synthesize adjacent (consecutively occurring) γ-carboxyglutamic acid residues. More preferably, the peptides are free of γ-carboxyglutamic acid residues. Such synthetic peptides have many advantages over the use of native amino acids 1-48 of the Gla domain of factor IXa or the entire factor IXa chain.

Historically, portions of the factor IXa chain comprising a γ-carboxyglutamic acid residue have not been readily produced by synthetic techniques. Such peptides are usually made by recombinant DNA techniques, which are expensive and time consuming. Further, the native factor IXa Gla domain requires calcium ions to interact with its Gla residues to assume the proper three-dimensional shape. Synthetic peptides which are conformationally constrained without Gla residues do not require the presence of calcium ions for proper three-dimensional shape. Also, shorter synthetic peptides may be more soluble and less immunogenic than larger proteins.

As used herein, "peptide" refers to a linear series of no more than about seventy-five (75) amino acid residues connected to one another by peptide bonds between the alpha-amino groups and carboxy groups of adjacent amino acid residues. Additional covalent bonds between portions of the peptide are also present to restrain the conformation of the

molecule, such as amide and disulfide bonds. The term "synthetic peptide" means a chemically derived chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof.

The term "homology" as describing the relationship between two amino acid sequences means the extent to which the sequences, viewed from the N-terminal to the C-terminal direction, have segments of their sequences which are identical and which occur in the same N-terminal to C-terminal order in the overall sequence. The synthetic peptides according to the invention have an amino acid sequence which is the same as that of the native amino acid sequence, but for inserted, deleted, or interchanged (one or more amino acids is substituted for the same number of other amino acids) portions.

The degree of amino acid sequence homology between the amino acid sequence of a synthetic peptide according to the invention and that of the native peptide is expressed as a percentage. This percentage is obtained by determining the number of amino acids in the sequence of the synthetic peptide which occur in segments that are identical to segments of the native amino acid sequence and which occur in the same N-terminal to C-terminal order as the native segments, divided by the total number of amino acids in the native sequence.

A "substantial amino acid sequence homology" is any amino acid sequence homology greater that 30 percent. Preferably the homology is greater than 80 percent, most preferably greater than 90 percent.

Peptides of the present invention include any analog, fragment or chemical derivative of the peptides capable of inhibiting the binding of factor IXa binding to platelets. The term "analog" includes any peptide having substantial amino acid sequence homology to the peptides of the invention in which one or more amino acids have been substituted with other amino acids, and the substituted amino acids allow or require the peptide to assume the equilibrium conformation of the domain of the parent protein. Often, cysteine, lysine and glutamic acid will be used for their side chains which can form covalent linkages to restrict the conformation of

a peptide. In addition, conservative amino acid changes may be made which do not alter the biological function of the peptide. For instance, one polar amino acid, such as glycine, may be substituted for another polar amino acid; or one acidic amino acid, such as aspartic acid may be substituted for another acidic amino acid, such as glutamic acid; or a basic amino acid, such as lysine, arginine or histidine may be substituted for another basic amino acid; or a non-polar amino acid, such as alanine, leucine or isoleucine may be substituted for another non-polar amino acid.

The term "analog" shall also include any peptide which has one or more amino acids deleted from or added to an amino acid sequence identical to that of the native fragment of the amino acid sequence in the Gla-domain in the factor IX chain, but which still retains a substantial amino acid sequence homology to the platelet binding site on factor IXa, as well as the ability to inhibit the binding of factor IXa to platelets. Further, the preferred peptides do not contain adjacent γ-carboxy-glutamic acid amino acid residues, and more preferably have no γ-carboxyglutamic acid amino acid residues.

The term "fragment" shall refer to any shorter version of the peptides identified herein having at least five amino acid residues, wherein the fragment is a synthetic peptide which is capable of inhibiting the binding of factor IXa to platelets.

The three-letter symbols used to represent the amino acid residues in the peptides of the present invention are those symbols commonly used in the art. The amino acid residues are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form may be substituted for any L-amino acid, as long as the desired functional property of inhibition of factor IXa-induced factor IX activation is retained by the peptide. The three-letter symbols used herein refer to the following amino acids: Ser is serine; Ile is isoleucine; Gln is glutamine; Phe is phenylalanine; His is histidine; Trp is tryptophan; Lys is lysine; Asn is asparagine; Leu is leucine; Gly is glycine; Thr is threonine;

Asp is aspartic acid; Arg is arginine; Gla is γ-carboxyglutamic acid; and Ala is alanine.

The peptides of the present invention may be prepared by any of the following known techniques. Conveniently, the peptides may be prepared using the solid-phase synthetic technique initially described by Merrifield, in J. Am. Chem. Soc. 15, 2149-2154 (1963). Other peptide synthesis techniques may be found, for example, in M. Bodanszky et al., Peptide Synthesis, John Wiley & Sons, 2d Ed. (1976); Kent and Clark-Lewis in Synthetic Peptides in Biology and Medicine, eds. Alitalo, K., Partanen, P. and Vakeri, A., (Elsevier Science Publishers, Amsterdam, 1985) p. 295-58; as well as other reference works known to those skilled in the art. A summary of peptide synthesis techniques may be found in J. Stuart and J.D. Young, Solid Phase Peptide Synthelia, Pierce Chemical Company, Rockford, IL (1984). The synthesis of peptides by solution methods may also be used, as described in The Proteins, vol II, 3d Ed., Neurath, H. et al., Eds., p. 105-237, Academic Press, New York, NY (1976). Appropriate protective groups for use in such syntheses will be found in the above texts as well as in J. F. W. McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, NY (1973). Of course, the present peptides may also be prepared by recombinant DNA techniques. But, such methods are not preferred because of the need for purification and subsequent chemical modifications to conformationally restrain the peptides.

In general, these synthetic methods involve the sequential addition of one or more amino acid residues or suitably protected amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively-removable protecting group. A different, selectively-removable protecting group is utilized for amino acids containing a reactive side group, such as lysine.

Using a solid phase synthesis as an example, the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then

selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted under conditions suitable for forming the amide linkage with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added amino acid residue, and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups (and solid support) are removed sequentially or concurrently, to provide the final peptide. The peptides of the invention are devoid of benzylated or methylbenzylated amino acids. Such protecting group moieties may be used in the course of synthesis, but they are removed before the peptides are used. Additional reactions may be necessary, as described elsewhere, to form intramolecular linkages to restrain conformation.

The peptides of the present invention generally contain at least five (5) amino acid residues and up to seventy-five (75) amino acid residues, preferably from about five (5) to about forty-five (45) amino acid residues, and as small as five (5) to about twenty (20) amino acids. The peptides may be linked to an additional sequence of amino acids either or both at the N-terminus and at the C-terminus, wherein the additional sequences are from 1-100 amino acids in length. Such additional amino acid sequences, or linker sequences, can be conveniently affixed to a detectable label or solid matrix, or carrier. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic acid and aspartic acid, or the like.

Any peptide of the present invention may be used in the form of a pharmaceutically acceptable salt. Suitable acids which are capable of forming salts with the peptides of the present invention include inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid and the like; and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric

acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid or the like.

Suitable bases capable of forming salts with the peptides of the present invention include inorganic bases such as sodium hydroxide, ammonium hydroxide, potassium hydroxide and the like; and organic bases such a mono-, di- and tri-alkyl and aryl amines (e.g., triethylamine, diisopropyl amine, methyl amine, dimethyl amine and the like) and optionally substituted ethanolamines (e.g., ethanolamine, diethanolamine and the like).

The present peptides are useful in a pharmaceutical composition for treatment to prevent intrinsic blood clotting. Such a pharmaceutical composition may be used to inhibit the binding of a platelet to factor IXa, or to inhibit the coagulant activity of factor IXa on the platelet surface. Thus, one or more of the synthetic peptides of the present invention may be present in a pharmaceutical composition in admixture with a pharmaceutically-acceptable carrier. The pharmaceutical composition may be compounded according to conventional pharmaceutical formulation techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., sublingual, rectal, nasal, oral or parenteral.

Compositions for oral dosage form may include any of the usual pharmaceutical media, such as, for example, water, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations (e.g., suspensions, elixirs and solutions) or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (e.g., powders, capsules and tablets). Controlled release forms may also be used. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar coated or enteric coated by standard techniques.

For compositions comprising the peptide according to the invention to be administered parenterally, the carrier

will usually comprise sterile water, although other ingredients to aid solubility or for preservation purposes may be included. Injectable suspensions may also be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed. The parenteral routes of administration may be intravenous injection, intramuscular injection or subcutaneous injection.

For intravenous administration, the peptides may be dissolved in an appropriate intravenous delivery vehicle containing physiologically compatible substances such as sodium chloride, glycine and the like, having a buffered pH compatible with physiologic conditions. Such intravenous delivery vehicles are known to those skilled in the art.

It is contemplated that the peptides of the present invention have utility as anticoagulant and/or antithrombotic agents. It is contemplated that the peptides may be administered to patients either at risk for developing arterial or venous thrombosis, or to patients with established thromboembolism to prevent extension of the thrombi. For example, it is contemplated that the peptides may find utility in the prevention and treatment of deep venous thrombosis and pulmonary embolism, treatment and prevention of cerebral vascular thromboembolism, the treatment and prevention of systemic arterial thrombosis and embolism, and the treatment and possibly the prophylaxis of established disseminated intravascular coagulation. Patients suffering from transient ischemic attacks are, in particular, at increased risk of brain damage through thrombus formation.

In particular, it is contemplated that the peptides of the present invention will find utility in the prevention of rethrombosis following lytic therapy. While lytic agents such as tissue plasminogen activator, urokinase and streptokinase have been utilized to dissolve vascular thrombi, their use is associated with a significant rate of rethrombosis, about 20-30%. This is because lytic therapy results in the exposure of a thrombogenic site, at the location of the prior thrombus. While lytic agents are effective in dissolving vascular thrombi, they offer no protection from clot reformation. The peptides of the present invention, by virtue of

their inhibition of the binding of factor IXa to the platelet surface and thus inhibition of factor IXa-induced activation of factor X on the platelet surface, are expected to possess substantial rethrombosis inhibiting activity. They may thus be administered as an adjuvant to lytic therapy to prevent reformation of dissolved vascular thrombi.

The peptides may be administered by any convenient means which will result in the delivery to the bloodstream of an amount effective to inhibit the binding of factor IXa to the platelet surface. Intravenous administration is presently contemplated as the preferred administration route. The amount administered will depend on the activity of the particular compound administered, which may be readily determined by those of ordinary skill in the art. The amount may also vary depending on the nature and extent of the lesion which is to be protected from rethrombosis; the size and weight of the patient; the route of administration, the age, sex and health of the patient; and other factors. Generally, the peptides may be administered in an amount sufficient to provide a plasma concentration in the range of from about 10^{-9} to about 10^{-5} M, more preferably in the range of from about 1×10^{-8} to about 5×10^{-6} M. Plasma concentrations higher or lower than these may be utilized, depending upon the activity of the particular compound being administered, and the nature of treatment.

It may be appreciated that a single bolus injection of 1 mg peptide per kilogram of treated subject body weight would achieve a maximum in vivo plasma concentration of 100 nM, assuming 100% recovery of drug. It is therefore contemplated that bolus administration will comprise a dosage of from about 0.1 mg to about 1 gram, per kilogram subject body weight. The bolus administration is most advantageously followed by a continuous infusion of peptide, as needed. The amount of peptide continuously infused depends on the approximate half-life of the peptide in the circulation. Those skilled in the art would, for any factor IXa platelet-binding-inhibiting peptide, be readily able to determine the half-life from routine experimentation.

The peptides of the invention are expected to inhibit intrinsic coagulation without affecting extrinsic coagulation. According to one exemplary treatment protocol, an amount of peptide shown effective by the in vitro assay described elsewhere herein, is administered to a patient by bolus administration and/or continuous infusion. The potency of the peptide and its clearance from the circulation is then monitored by drawing and assaying blood samples at timed intervals. The samples are assayed in parallel with control samples to compare clotting times. At the end of the evaluation period, the dosage is adjusted to provide the desired in vivo effect.

The following non-limiting examples serve to illustrate the practice of the invention.

Example 1

Computer Model

A structural model approximating the Factor IXa Gla domain (residues Tyr 1-Gly 48) was constructed using the computational chemistry package supplied by Molecular Simulations, Inc., Pasadena CA and a Silicon Graphics 4D 280 Parallel Processing Supercomputer. A description of the modeling package and methods has been previously published (Jameson, Nature 349, 465-466 (1989)). The coordinates from the bovine prothrombin a Gla crystal structure (Soriano-Garcia, et al. Biochem. 31 2554-2566 (1992) were used as a guideline. The amino acids of factor IXa Gla domain, residues 1-48, were substituted for the amino acids in the bovine prothrombin 1 Gla crystal structure based on sequence alignment of the prothrombin Gla domain and the factor IXa Gla domain. Thus, exchanges of the amino acids and resulting changes to coordinates were performed using the biopolymer module provided within the SYBYL computational chemistry package (Tripos Associates INC, St. Louis MO.). The Amber forcefield, as implemented in the SYBYL package, was utilized in all the subsequent calculations (Weiner, et al. J. Am. Chem. Soc. 106 765-784 (1984). Atomic parameters describing calcium and γ-carboxylated glutamic acid residues were added

to the force field table. The atomic properties of calcium (a transition state metal) are inadequately described within the force field tables to account for the coordination complexes formed between calcium atoms and the negative charges of the γ-carboxylated glutamic acids. Therefore, the distance-geometry measurements related to Ca^{2+} atoms and Gla residues in the prothrombin Gla domain coordinated complexes were held as restraints (not allowed to vary) during the modeling process of creating the factor IXa structure.

After all amino acid replacements were completed, and the additional atomic parameters and coordinates restraints added, the structure was energy minimized to convergence using a conjugate-gradient approach. Several ten picosecond high energy (900°K) dynamic runs (energy-dependent simulations of molecular motion) were used to dislodge inappropriate amino acid contacts. The structure was allowed to cool to 300°K over a 100 picosecond dynamics calculation, followed by minimization of the resulting structure. The newly energy minimized structure was then solvated with water (2 solvent shells were added to insure that all portions of the surface were adequately solvated) using the Silverware algorithm as implemented by SYBYL. The water-protein complex was again energy minimized prior to an energy-dependant simulation of molecular motion (t=100 picoseconds). A trajectory file, recorded during this entire dynamic run, indicated that after ~12 picoseconds of dynamics, the calculated backbone structure had stabilized, i.e., reached a low energy well. Thus, a stable low energy structure was obtained. Since a disulfide-bonded cysteine has an ideal bond length from α-carbon to α-carbon of ~5-6Å, we searched the factor IXa Gla domain structure model for ideal disulfide distances as well as for locations where a disulfide bond would not be expected to induce torsional stress. The calculated structure coordinates for the stable structure are set forth in Appendix 1.

Examples 2-4

Particularly useful peptide analogs which were derived using the techniques described herein comprise amino

acids corresponding to segments of the factor IXa Gla domain sequence residues 1-14. Conformationally restricted peptides corresponding to factor IXa amino acid residues 4-11 (SEQ ID NO: 2) and residues 4-8 (SEQ ID NO: 3) and residues 9-11 (SEQ ID NO: 4) were produced. To maintain the binding surfaces represented by amino acids Gly 4-Lys 5-Leu 6 and by Phe 9-Val 10-Gln 11 and eliminate γ-carboxyglutamic acid residues, the Gla residues at position 7 and 8 were changed to introduce a folding pattern similar to that present in the native structure. The predicted folding pattern of the putative structure was tested for its ability to mimic the structure observed in our model of the Gla domain of factor IXa. Finding satisfactory agreement, the peptides were synthesized according to conventional solid phase procedures on an Applied Biosystems 430A Peptide Synthesizer by a modification of the procedure described by Kent et al. in Synthetic Peptides in Biology and Medicine (Elsevier Science Publishers, Amsterdam, pp. 29-58 (1985)), in which dimethylformamide replaced methylene chloride in the routine wash cycles. The synthesis was carried out using a paramethylbenzhydrylamine resin (United States Biochemical Corp., Cleveland, OH). The solvents and protected amino acids were synthesis grade biotechnology products purchased from Fischer Scientific Co., Pittsburgh, PA. The resulting peptides were folded into a three-dimensional constrained conformation in a separate chemical reaction step after the peptide was purified as follows.

The peptides were each dissolved in deionized water as a 0.1 mg/ml solution in a flask containing a stir bar. The pH was adjusted to 8.5 with NH_4OH and each of the three solutions were allowed to stir at 5°C for at least three days. Each of the resulting solutions was lyophilized.

The folded peptides were examined by both reverse phase and gel filtration high performance liquid chromatography (HPLC). The HPLC system was the Waters 600 Gradient Module, Model 740 Data Module, Model 46K Universal Injector and Lambda-Max Model 481 Detector. Reverse phase chromatography was performed using a Waters C8 μBondapak Column equilibrated with 0.1% (V/V) trifluoroacetic acid. The column was eluted with a linear gradient of aqueous acetonitrile

containing 0.1% trifluoroacetic acid with a detector set at a wavelength of 206 nm. Gel filtration of the peptides was also carried out using a Waters Protein-Pak 60 column which was run isocratically with 0.1% (V/V) trifluoroacetic in 20% acetonitrile. Each of the three folded peptides demonstrated a single homogenous peak with a retention time identical to the corresponding unfolded peptide. This indicates the presence of a single homogeneous mixture for each folded peptide, and not a mixed population of diverse polymers.

Example 5

Effect of Gla-Domain Derived Peptide on the Binding of Factor IXa to Platelets

A. Purification of Human Coagulation Factors.

Human coagulation proteins, including factor IX, factor IXa, factor VIII, factor X and α-thrombin, were purified, assayed and characterized as previously published (Ahmad, et al. J. Biol. Chem. 264 3244-3251, (1989). The conditions used for activation of factor VIII with human α-thrombin were identical to those previously published. All proteins were >98% pure as determined by polyacrylamide slab gel electrophoresis. Factor IX molecule was radiolabeled with ^{125}I by the iodogen method as previously described (Ahmad, et al. supra). Specific radioactivities of all proteins were in the range of 2.0-2.5 x 10^6 cpm/μg. Activation of factor IX by purified factor XIa was carried out as described by Ahmad, et al. supra. The p-aminobenzamidine fluorescence assay was employed to quantitatively examine the activation of factor IX as previously reported by Lin, et al. J. Biol. Chem. 265 144-150 (1990).

B. Purified IXa

Purified IXa was labeled with ^{125}I by a minor modification (Sinha et al., J. Biol. Chem. 260 10714-10719 (1985)) of the iodogen method to a specific activity of 5 x 10^6 cpm/mg. The radiolabeled protein retained >90% of its biological activity compared with unlabeled factor IX.

C. Assay of Factor IXa Binding to Platelets

All incubations were performed at 37°C without stirring the reaction mixture. Gel-filtered platelets (3-4 x 10^8/ml) in calcium-free 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) Tyrode's buffer, pH 7.4, were incubated at 37°C in a 1.5 ml Eppendorf plastic centrifuge tube with mixtures of unlabeled and radiolabeled factor IXa (0.1-20 nM), calcium chloride (5 mM), and human α-thrombin (0.1 U/ml) in the presence or absence of factor X (1.5 μM) and thrombin activated factor VIII (2 U/ml) as detailed previously (Ahmad, et al., supra). At various times after the addition of the platelet stimulus, aliquots were removed and centrifuged through a mixture of silicone oils as described in Greengard et al., Biochem., 25, 3884-3890 (1986). The data were analyzed and the number of binding sites and dissociation constants (K_d) were calculated from the means of three independent determinations, each done in duplicate, as previously described (Ahmad, et al., supra) using a Mac Plus Computer and the LIGAND Program as modified by G.A. McPherson (Elsevier Science Publishers BV, The Netherlands, 1985). Total binding was not corrected for any nonsaturable component. More than 86% of the platelets were sedimented under these conditions.

D. Effect of Peptides on Factor IXa-Platelet Binding.

Platelets were incubated as described in Example 5C, above, with various concentrations of synthetic peptides, factor IXa or buffer, followed by incubation with radiolabeled factor IXa. After 20 minutes, samples were centrifuged. Binding of ^{125}I-factor IXa was compared to control binding in the absence of competing synthetic peptides or unlabelled factor IXa.

The IC_{50} method of Cha, Biochem. Pharmacol. 24 2177-2185 (1975) was used to determine the inhibitor constants K_i as previously described (Sinha et al., Biochem. 26 3768-3775 (1987)). In the case of classical competitive inhibition, IC_{50} (total inhibitor concentration at which the enzyme

reaction velocity is 50% of the uninhibited reaction) is related to the substrate concentration as follows,

$$I_{50} = 1/2\ Et + K_i + K_iS/K_m$$

where Et it the total enzyme concentration and S is the substrate concentration. K_i was thus determined from the plot of IC_{50} vs S. The results are set forth in Table 1:

TABLE 1

Competing Factor IXa or Gla Domain Peptide	K_i of Peptide Inhibition of Factor of IXa Binding to Platelet
Factor IXa	0.5×10^{-9}
SEQ ID NO:2	3.5×10^{-8}
SEQ ID NO:3	1.0×10^{-6}
SEQ ID NO:4	1.0×10^{-5}

E. Synergism Between SEQ ID NO: 3 and SEQ ID NO: 4

The above binding assay was repeated with a mixture comprising equimolar amounts of the two peptides SEQ ID NO:3 and SEQ ID NO:4. These peptides, as shown in Table 1, separately displayed inhibitory activities in the binding assay with K_i's of 10^{-6} and 10^{-5} M respectively. When tested together in equimolar concentrations, the two peptides demonstrated striking synergism with a K_i of 1×10^{-7} see table 2, below. The concentrations of the combined peptides required to inhibit factor IXa binding to platelets 50% were 50-fold lower than expected on the basis of their inhibitory activities when used alone.

TABLE 2

Competing Factor IXa or Domain Peptide	K_i of Peptide Inhibition of Factor IXa Gla Binding to Platelet
SEQ ID NO:3 + SEQ ID NO:4*	1.0×10^{-7}

*Two peptides added together at equimolar concentration.

Example 6

Anti-Coagulant Effect of Gla Domain-Derived Peptides

The peptides derived from the factor IXa Gla domain are assayed for possible inhibitory effects on blood coagulation as follows. Phospholipids can substitute for platelets in most coagulation reactions. Thus, parallel assays are run with the peptides to determine whether their inhibitory effects were specific for their interaction with platelets.

The experimental protocol involves the assay of factor IXa activity by minor modifications of the method according to Scott et al., Blood 63 42-50 (1984). This assay determines the kaolin-activated partial thromboplastin time (Proctor et al., Am. J. Clin. Pathol. 36 212-219 (1961)).

APPENDIX 1

Factor IX GLA-Domain Brookhaven Format

ATOM	1	N	TYR	1	-10.697	-8.531	14.159	1.00	0.00
ATOM	2	CA	TYR	1	-11.866	-8.660	13.303	1.00	0.00
ATOM	3	C	TYR	1	-13.118	-9.144	14.040	1.00	0.00
ATOM	4	O	TYR	1	-13.534	-10.290	13.864	1.00	0.00
ATOM	5	CB	TYR	1	-11.549	-9.458	12.028	1.00	0.00
ATOM	6	CG	TYR	1	-11.242	-10.940	12.147	1.00	0.00
ATOM	7	CD1	TYR	1	-10.541	-11.442	13.258	1.00	0.00
ATOM	8	CD2	TYR	1	-11.605	-11.812	11.102	1.00	0.00
ATOM	9	CE1	TYR	1	-10.178	-12.797	13.309	1.00	0.00
ATOM	10	CE2	TYR	1	-11.266	-13.177	11.164	1.00	0.00
ATOM	11	CZ	TYR	1	-10.548	-13.662	12.271	1.00	0.00
ATOM	12	OH	TYR	1	-10.179	-14.973	12.336	1.00	0.00
ATOM	13	H	TYR	1	-10.473	-7.617	14.557	1.00	0.00
ATOM	14	HH	TYR	1	-9.713	-15.124	13.163	1.00	0.00
ATOM	15	N	ASN	2	-13.741	-8.252	14.816	1.00	0.00
ATOM	16	CA	ASN	2	-15.060	-8.399	15.428	1.00	0.00
ATOM	17	C	ASN	2	-15.418	-9.827	15.829	1.00	0.00
ATOM	18	O	ASN	2	-16.231	-10.473	15.163	1.00	0.00
ATOM	19	CB	ASN	2	-16.098	-7.826	14.467	1.00	0.00
ATOM	20	CG	ASN	2	-16.930	-6.753	15.141	1.00	0.00
ATOM	21	OD1	ASN	2	-18.131	-6.939	15.340	1.00	0.00
ATOM	22	ND2	ASN	2	-16.299	-5.638	15.480	1.00	0.00
ATOM	23	H	ASN	2	-13.376	-7.295	14.830	1.00	0.00
ATOM	24	HD21	ASN	2	-15.299	-5.531	15.251	1.00	0.00
ATOM	25	HD22	ASN	2	-16.794	-4.854	15.921	1.00	0.00
ATOM	26	N	SER	3	-14.811	-10.348	16.895	1.00	0.00
ATOM	27	CA	SER	3	-14.828	-11.785	17.098	1.00	0.00
ATOM	28	C	SER	3	-15.369	-12.259	18.443	1.00	0.00
ATOM	29	O	SER	3	-15.958	-13.340	18.501	1.00	0.00
ATOM	30	CB	SER	3	-13.481	-12.406	16.738	1.00	0.00
ATOM	31	OG	SER	3	-13.431	-12.634	15.345	1.00	0.00
ATOM	32	H	SER	3	-14.286	-9.752	17.544	1.00	0.00
ATOM	33	HG	SER	3	-13.369	-11.776	14.902	1.00	0.00
ATOM	34	N	GLY	4	-15.182	-11.498	19.521	1.00	0.00
ATOM	35	CA	GLY	4	-15.652	-11.915	20.830	1.00	0.00
ATOM	36	C	GLY	4	-17.142	-11.640	20.980	1.00	0.00
ATOM	37	O	GLY	4	-17.981	-12.460	20.606	1.00	0.00
ATOM	38	H	GLY	4	-14.707	-10.595	19.430	1.00	0.00
ATOM	39	N	LYS	5	-17.459	-10.483	21.551	1.00	0.00
ATOM	40	CA	LYS	5	-18.791	-9.933	21.668	1.00	0.00
ATOM	41	C	LYS	5	-18.618	-8.443	21.901	1.00	0.00
ATOM	42	O	LYS	5	-18.469	-8.016	23.044	1.00	0.00
ATOM	43	CB	LYS	5	-19.567	-10.573	22.827	1.00	0.00
ATOM	44	CG	LYS	5	-20.956	-9.935	22.966	1.00	0.00
ATOM	45	CD	LYS	5	-21.027	-9.029	24.199	1.00	0.00
ATOM	46	CE	LYS	5	-21.537	-7.633	23.830	1.00	0.00
ATOM	47	NZ	LYS	5	-20.491	-6.611	24.023	1.00	0.00
ATOM	48	H	LYS	5	-16.688	-9.885	21.863	1.00	0.00
ATOM	49	HZ3	LYS	5	-19.620	-6.937	23.606	1.00	0.00
ATOM	50	HZ2	LYS	5	-20.315	-6.443	25.003	1.00	0.00
ATOM	51	HZ1	LYS	5	-20.743	-5.747	23.559	1.00	0.00
ATOM	52	N	LEU	6	-18.696	-7.655	20.831	1.00	0.00
ATOM	53	CA	LEU	6	-18.738	-6.203	20.902	1.00	0.00
ATOM	54	C	LEU	6	-17.598	-5.627	21.753	1.00	0.00
ATOM	55	O	LEU	6	-17.817	-5.228	22.904	1.00	0.00
ATOM	56	CB	LEU	6	-20.128	-5.724	21.335	1.00	0.00
ATOM	57	CG	LEU	6	-21.211	-6.052	20.310	1.00	0.00
ATOM	58	CD1	LEU	6	-21.768	-7.449	20.560	1.00	0.00

ATOM	59	CD2	LEU	6	-22.354	-5.054	20.465	1.00	0.00
ATOM	60	H	LEU	6	-18.720	-8.074	19.913	1.00	0.00
ATOM	61	N	GLA	7	-16.395	-5.658	21.175	1.00	0.00
ATOM	62	CA	GLA	7	-15.117	-5.304	21.775	1.00	0.00
ATOM	63	C	GLA	7	-14.847	-3.796	21.765	1.00	0.00
ATOM	64	O	GLA	7	-14.492	-3.235	22.805	1.00	0.00
ATOM	65	CB	GLA	7	-14.009	-6.085	21.062	1.00	0.00
ATOM	66	CG	GLA	7	-14.404	-7.560	21.009	1.00	0.00
ATOM	67	CD	GLA	7	-14.578	-8.149	22.418	1.00	0.00
ATOM	68	OE2	GLA	7	-15.392	-9.092	22.568	1.00	0.00
ATOM	69	OE1	GLA	7	-13.930	-7.654	23.371	1.00	0.00
ATOM	70	C12	GLA	7	-13.527	-8.425	20.104	1.00	0.00
ATOM	71	O13	GLA	7	-12.473	-8.861	20.615	1.00	0.00
ATOM	72	O14	GLA	7	-14.111	-9.012	19.157	1.00	0.00
ATOM	73	N	GLA	8	-15.024	-3.168	20.597	1.00	0.00
ATOM	74	CA	GLA	8	-14.962	-1.745	20.266	1.00	0.00
ATOM	75	C	GLA	8	-15.037	-0.823	21.501	1.00	0.00
ATOM	76	O	GLA	8	-14.054	-0.183	21.891	1.00	0.00
ATOM	77	CB	GLA	8	-15.976	-1.433	19.135	1.00	0.00
ATOM	78	CG	GLA	8	-16.847	-2.658	18.750	1.00	0.00
ATOM	79	CD	GLA	8	-17.650	-2.577	17.450	1.00	0.00
ATOM	80	OE2	GLA	8	-17.498	-3.505	16.619	1.00	0.00
ATOM	81	OE1	GLA	8	-18.417	-1.600	17.274	1.00	0.00
ATOM	82	C12	GLA	8	-17.872	-2.974	19.853	1.00	0.00
ATOM	83	O13	GLA	8	-18.052	-2.102	20.732	1.00	0.00
ATOM	84	O14	GLA	8	-18.758	-3.824	19.610	1.00	0.00
ATOM	85	N	PHE	9	-16.201	-0.829	22.159	1.00	0.00
ATOM	86	CA	PHE	9	-16.568	-0.169	23.409	1.00	0.00
ATOM	87	C	PHE	9	-15.422	-0.007	24.413	1.00	0.00
ATOM	88	O	PHE	9	-15.319	1.033	25.065	1.00	0.00
ATOM	89	CB	PHE	9	-17.720	-0.962	24.038	1.00	0.00
ATOM	90	CG	PHE	9	-18.653	-0.212	24.967	1.00	0.00
ATOM	91	CD1	PHE	9	-19.800	0.408	24.440	1.00	0.00
ATOM	92	CD2	PHE	9	-18.550	-0.407	26.357	1.00	0.00
ATOM	93	CE1	PHE	9	-20.803	0.891	25.300	1.00	0.00
ATOM	94	CE2	PHE	9	-19.552	0.074	27.219	1.00	0.00
ATOM	95	CZ	PHE	9	-20.678	0.729	26.691	1.00	0.00
ATOM	96	H	PHE	9	-16.926	-1.385	21.708	1.00	0.00
ATOM	97	N	VAL	10	-14.586	-1.035	24.581	1.00	0.00
ATOM	98	CA	VAL	10	-13.539	-1.062	25.591	1.00	0.00
ATOM	99	C	VAL	10	-12.507	0.055	25.443	1.00	0.00
ATOM	100	O	VAL	10	-12.127	0.667	26.444	1.00	0.00
ATOM	101	CB	VAL	10	-12.875	-2.442	25.622	1.00	0.00
ATOM	102	CG1	VAL	10	-11.606	-2.460	26.474	1.00	0.00
ATOM	103	CG2	VAL	10	-13.851	-3.471	26.185	1.00	0.00
ATOM	104	H	VAL	10	-14.691	-1.839	23.965	1.00	0.00
ATOM	105	N	GLN	11	-11.986	0.270	24.233	1.00	0.00
ATOM	106	CA	GLN	11	-10.729	0.986	24.090	1.00	0.00
ATOM	107	C	GLN	11	-9.892	0.446	22.933	1.00	0.00
ATOM	108	O	GLN	11	-9.274	-0.610	23.067	1.00	0.00
ATOM	109	CB	GLN	11	-10.937	2.504	24.086	1.00	0.00
ATOM	110	CG	GLN	11	-9.610	3.273	24.139	1.00	0.00
ATOM	111	CD	GLN	11	-8.606	2.697	25.133	1.00	0.00
ATOM	112	OE1	GLN	11	-8.701	2.923	26.336	1.00	0.00
ATOM	113	NE2	GLN	11	-7.608	1.977	24.629	1.00	0.00
ATOM	114	HE21	GLN	11	-7.525	1.844	23.620	1.00	0.00
ATOM	115	HE22	GLN	11	-6.939	1.549	25.245	1.00	0.00
ATOM	116	H	GLN	11	-12.390	-0.200	23.427	1.00	0.00
ATOM	117	N	GLY	12	-9.853	1.184	21.823	1.00	0.00
ATOM	118	CA	GLY	12	-9.056	0.830	20.662	1.00	0.00
ATOM	119	C	GLY	12	-7.558	0.862	20.953	1.00	0.00
ATOM	120	O	GLY	12	-7.112	1.557	21.875	1.00	0.00
ATOM	121	H	GLY	12	-10.399	2.030	21.771	1.00	0.00
ATOM	122	N	ASN	13	-6.800	0.117	20.150	1.00	0.00
ATOM	123	CA	ASN	13	-5.353	-0.055	20.184	1.00	0.00
ATOM	124	C	ASN	13	-5.005	-1.227	19.272	1.00	0.00
ATOM	125	O	ASN	13	-5.491	-2.331	19.498	1.00	0.00
ATOM	126	CB	ASN	13	-4.860	-0.249	21.628	1.00	0.00
ATOM	127	CG	ASN	13	-3.572	-1.054	21.818	1.00	0.00
ATOM	128	OD1	ASN	13	-3.447	-1.772	22.807	1.00	0.00
ATOM	129	ND2	ASN	13	-2.577	-0.894	20.947	1.00	0.00
ATOM	130	H	ASN	13	-7.316	-0.461	19.478	1.00	0.00
ATOM	131	HD21	ASN	13	-2.670	-0.316	20.131	1.00	0.00

ATOM	132	HD22	ASN	13	-1.755	-1.512	21.010	1.00	0.00
ATOM	133	N	LEU	14	-4.171	-0.955	18.255	1.00	0.00
ATOM	134	CA	LEU	14	-3.688	-1.915	17.261	1.00	0.00
ATOM	135	C	LEU	14	-3.657	-3.367	17.754	1.00	0.00
ATOM	136	O	LEU	14	-4.352	-4.216	17.195	1.00	0.00
ATOM	137	CB	LEU	14	-2.311	-1.485	16.749	1.00	0.00
ATOM	138	CG	LEU	14	-2.356	-0.154	16.002	1.00	0.00
ATOM	139	CD1	LEU	14	-0.968	0.159	15.450	1.00	0.00
ATOM	140	CD2	LEU	14	-3.337	-0.239	14.840	1.00	0.00
ATOM	141	H	LEU	14	-3.884	0.002	18.124	1.00	0.00
ATOM	142	N	GLA	15	-2.867	-3.632	18.801	1.00	0.00
ATOM	143	CA	GLA	15	-2.834	-4.905	19.509	1.00	0.00
ATOM	144	C	GLA	15	-4.177	-5.153	20.230	1.00	0.00
ATOM	145	O	GLA	15	-4.275	-4.903	21.434	1.00	0.00
ATOM	146	CB	GLA	15	-1.639	-4.939	20.488	1.00	0.00
ATOM	147	CG	GLA	15	-0.238	-4.613	19.917	1.00	0.00
ATOM	148	CD	GLA	15	0.906	-5.357	20.626	1.00	0.00
ATOM	149	OE2	GLA	15	1.867	-5.753	19.922	1.00	0.00
ATOM	150	OE1	GLA	15	0.836	-5.528	21.868	1.00	0.00
ATOM	151	C12	GLA	15	0.033	-3.102	19.838	1.00	0.00
ATOM	152	O13	GLA	15	-0.336	-2.374	20.794	1.00	0.00
ATOM	153	O14	GLA	15	0.577	-2.657	18.799	1.00	0.00
ATOM	154	N	ARG	16	-5.191	-5.649	19.502	1.00	0.00
ATOM	155	CA	ARG	16	-6.532	-5.992	19.987	1.00	0.00
ATOM	156	C	ARG	16	-7.558	-6.110	18.854	1.00	0.00
ATOM	157	O	ARG	16	-7.759	-7.204	18.334	1.00	0.00
ATOM	158	CB	ARG	16	-7.034	-5.092	21.122	1.00	0.00
ATOM	159	CG	ARG	16	-7.018	-5.851	22.453	1.00	0.00
ATOM	160	CD	ARG	16	-8.285	-5.633	23.286	1.00	0.00
ATOM	161	NE	ARG	16	-8.870	-4.309	23.060	1.00	0.00
ATOM	162	CZ	ARG	16	-10.047	-4.118	22.449	1.00	0.00
ATOM	163	NH1	ARG	16	-10.530	-2.881	22.320	1.00	0.00
ATOM	164	NH2	ARG	16	-10.714	-5.180	21.992	1.00	0.00
ATOM	165	H	ARG	16	-4.994	-5.865	18.525	1.00	0.00
ATOM	166	HE	ARG	16	-8.284	-3.505	23.237	1.00	0.00
ATOM	167	HH11	ARG	16	-9.839	-2.126	22.252	1.00	0.00
ATOM	168	HH12	ARG	16	-11.331	-2.740	21.692	1.00	0.00
ATOM	169	HH21	ARG	16	-10.302	-6.097	22.063	1.00	0.00
ATOM	170	HH22	ARG	16	-11.343	-5.026	21.191	1.00	0.00
ATOM	171	N	GLA	17	-8.220	-5.005	18.481	1.00	0.00
ATOM	172	CA	GLA	17	-9.289	-5.039	17.484	1.00	0.00
ATOM	173	C	GLA	17	-8.727	-5.468	16.127	1.00	0.00
ATOM	174	O	GLA	17	-9.222	-6.378	15.461	1.00	0.00
ATOM	175	CB	GLA	17	-9.929	-3.654	17.303	1.00	0.00
ATOM	176	CG	GLA	17	-10.214	-2.800	18.545	1.00	0.00
ATOM	177	CD	GLA	17	-11.312	-3.289	19.480	1.00	0.00
ATOM	178	OE2	GLA	17	-11.924	-2.434	20.161	1.00	0.00
ATOM	179	OE1	GLA	17	-11.487	-4.519	19.618	1.00	0.00
ATOM	180	C12	GLA	17	-8.978	-2.141	19.178	1.00	0.00
ATOM	181	O13	GLA	17	-8.854	-2.142	20.427	1.00	0.00
ATOM	182	O14	GLA	17	-8.409	-1.276	18.471	1.00	0.00
ATOM	183	N	CYS	18	-7.702	-4.728	15.705	1.00	0.00
ATOM	184	CA	CYS	18	-7.161	-4.804	14.366	1.00	0.00
ATOM	185	C	CYS	18	-6.151	-5.934	14.243	1.00	0.00
ATOM	186	O	CYS	18	-6.316	-6.822	13.402	1.00	0.00
ATOM	187	CB	CYS	18	-6.544	-3.459	13.980	1.00	0.00
ATOM	188	SG	CYS	18	-7.601	-2.436	12.927	1.00	0.00
ATOM	189	H	CYS	18	-7.377	-3.998	16.330	1.00	0.00
ATOM	190	LPG2	CYS	18	-7.097	-2.089	12.623	1.00	0.00
ATOM	191	LPG1	CYS	18	-8.174	-2.522	13.279	1.00	0.00
ATOM	192	N	MET	19	-5.092	-5.872	15.053	1.00	0.00
ATOM	193	CA	MET	19	-3.981	-6.808	15.042	1.00	0.00
ATOM	194	C	MET	19	-4.035	-7.725	16.268	1.00	0.00
ATOM	195	O	MET	19	-4.662	-7.385	17.274	1.00	0.00
ATOM	196	CB	MET	19	-2.663	-6.032	14.954	1.00	0.00
ATOM	197	CG	MET	19	-2.652	-5.163	13.695	1.00	0.00
ATOM	198	SD	MET	19	-1.960	-3.497	13.674	1.00	0.00
ATOM	199	CE	MET	19	-0.221	-3.921	14.146	1.00	0.00
ATOM	200	LPD1	MET	19	-1.905	-3.361	13.210	1.00	0.00
ATOM	201	LPD2	MET	19	-2.126	-3.410	14.525	1.00	0.00
ATOM	202	H	MET	19	-5.060	-5.159	15.772	1.00	0.00
ATOM	203	N	GLA	20	-3.382	-8.888	16.136	1.00	0.00
ATOM	204	CA	GLA	20	-3.758	-10.161	16.743	1.00	0.00

ATOM	205	C	GLA	20	-5.042	-10.721	16.097	1.00	0.00
ATOM	206	O	GLA	20	-4.982	-11.760	15.433	1.00	0.00
ATOM	207	CB	GLA	20	-3.664	-10.168	18.281	1.00	0.00
ATOM	208	CG	GLA	20	-2.450	-10.969	18.810	1.00	0.00
ATOM	209	CD	GLA	20	-2.763	-12.065	19.841	1.00	0.00
ATOM	210	OE2	GLA	20	-2.315	-13.214	19.611	1.00	0.00
ATOM	211	OE1	GLA	20	-3.451	-11.782	20.850	1.00	0.00
ATOM	212	C12	GLA	20	-1.168	-10.174	19.072	1.00	0.00
ATOM	213	O13	GLA	20	-1.175	-9.327	19.992	1.00	0.00
ATOM	214	O14	GLA	20	-0.129	-10.585	18.502	1.00	0.00
ATOM	215	N	GLA	21	-6.162	-9.983	16.168	1.00	0.00
ATOM	216	CA	GLA	21	-7.341	-10.046	15.297	1.00	0.00
ATOM	217	C	GLA	21	-6.999	-9.867	13.802	1.00	0.00
ATOM	218	O	GLA	21	-5.848	-10.041	13.400	1.00	0.00
ATOM	219	CB	GLA	21	-8.253	-11.259	15.569	1.00	0.00
ATOM	220	CG	GLA	21	-8.184	-11.880	16.974	1.00	0.00
ATOM	221	CD	GLA	21	-7.487	-13.248	16.947	1.00	0.00
ATOM	222	OE2	GLA	21	-7.493	-13.897	15.869	1.00	0.00
ATOM	223	OE1	GLA	21	-6.920	-13.662	17.982	1.00	0.00
ATOM	224	C12	GLA	21	-9.531	-11.948	17.694	1.00	0.00
ATOM	225	O13	GLA	21	-10.332	-12.840	17.334	1.00	0.00
ATOM	226	O14	GLA	21	-9.589	-11.397	18.814	1.00	0.00
ATOM	227	N	LYS	22	-7.997	-9.618	12.950	1.00	0.00
ATOM	228	CA	LYS	22	-7.795	-9.380	11.521	1.00	0.00
ATOM	229	C	LYS	22	-8.766	-8.335	10.972	1.00	0.00
ATOM	230	O	LYS	22	-9.564	-8.626	10.080	1.00	0.00
ATOM	231	CB	LYS	22	-7.764	-10.654	10.659	1.00	0.00
ATOM	232	CG	LYS	22	-7.817	-11.974	11.419	1.00	0.00
ATOM	233	CD	LYS	22	-6.520	-12.759	11.232	1.00	0.00
ATOM	234	CE	LYS	22	-6.267	-13.601	12.478	1.00	0.00
ATOM	235	NZ	LYS	22	-6.595	-12.843	13.695	1.00	0.00
ATOM	236	H	LYS	22	-8.928	-9.489	13.326	1.00	0.00
ATOM	237	HZ3	LYS	22	-6.694	-13.437	14.515	1.00	0.00
ATOM	238	HZ2	LYS	22	-7.484	-12.376	13.599	1.00	0.00
ATOM	239	HZ1	LYS	22	-5.890	-12.148	13.943	1.00	0.00
ATOM	240	N	CYS	23	-8.694	-7.118	11.513	1.00	0.00
ATOM	241	CA	CYS	23	-9.616	-6.030	11.190	1.00	0.00
ATOM	242	C	CYS	23	-9.612	-5.617	9.712	1.00	0.00
ATOM	243	O	CYS	23	-8.739	-6.021	8.944	1.00	0.00
ATOM	244	CB	CYS	23	-9.285	-4.825	12.060	1.00	0.00
ATOM	245	SG	CYS	23	-8.054	-3.704	11.349	1.00	0.00
ATOM	246	H	CYS	23	-7.993	-6.956	12.228	1.00	0.00
ATOM	247	LPG2	CYS	23	-8.493	-3.298	11.026	1.00	0.00
ATOM	248	LPG1	CYS	23	-7.520	-4.124	11.414	1.00	0.00
ATOM	249	N	SER	24	-10.572	-4.771	9.320	1.00	0.00
ATOM	250	CA	SER	24	-10.554	-4.199	7.987	1.00	0.00
ATOM	251	C	SER	24	-10.661	-2.671	7.973	1.00	0.00
ATOM	252	O	SER	24	-9.683	-1.977	8.242	1.00	0.00
ATOM	253	CB	SER	24	-11.527	-4.927	7.056	1.00	0.00
ATOM	254	OG	SER	24	-12.843	-4.894	7.570	1.00	0.00
ATOM	255	H	SER	24	-11.251	-4.437	9.995	1.00	0.00
ATOM	256	HG	SER	24	-12.824	-5.171	8.494	1.00	0.00
ATOM	257	N	PHE	25	-11.840	-2.151	7.627	1.00	0.00
ATOM	258	CA	PHE	25	-12.046	-0.719	7.466	1.00	0.00
ATOM	259	C	PHE	25	-12.551	-0.075	8.763	1.00	0.00
ATOM	260	O	PHE	25	-11.746	0.457	9.530	1.00	0.00
ATOM	261	CB	PHE	25	-12.958	-0.438	6.267	1.00	0.00
ATOM	262	CG	PHE	25	-12.261	-0.191	4.945	1.00	0.00
ATOM	263	CD1	PHE	25	-12.342	-1.153	3.920	1.00	0.00
ATOM	264	CD2	PHE	25	-11.675	1.061	4.684	1.00	0.00
ATOM	265	CE1	PHE	25	-11.801	-0.882	2.650	1.00	0.00
ATOM	266	CE2	PHE	25	-11.131	1.331	3.415	1.00	0.00
ATOM	267	CZ	PHE	25	-11.198	0.362	2.399	1.00	0.00
ATOM	268	H	PHE	25	-12.615	-2.785	7.465	1.00	0.00
ATOM	269	N	GLA	26	-13.874	-0.118	8.987	1.00	0.00
ATOM	270	CA	GLA	26	-14.580	0.472	10.129	1.00	0.00
ATOM	271	C	GLA	26	-13.804	0.352	11.451	1.00	0.00
ATOM	272	O	GLA	26	-13.464	1.374	12.047	1.00	0.00
ATOM	273	CB	GLA	26	-16.020	-0.065	10.183	1.00	0.00
ATOM	274	CG	GLA	26	-17.125	0.934	10.580	1.00	0.00
ATOM	275	CD	GLA	26	-16.852	2.364	10.080	1.00	0.00
ATOM	276	OE2	GLA	26	-16.884	2.592	8.848	1.00	0.00
ATOM	277	OE1	GLA	26	-16.591	3.246	10.930	1.00	0.00

ATOM	278	C12	GLA	26	-18.534	0.421	10.213	1.00	0.00
ATOM	279	O13	GLA	26	-19.528	0.859	10.843	1.00	0.00
ATOM	280	O14	GLA	26	-18.645	-0.349	9.230	1.00	0.00
ATOM	281	N	GLA	27	-13.439	-0.872	11.864	1.00	0.00
ATOM	282	CA	GLA	27	-12.679	-1.173	13.081	1.00	0.00
ATOM	283	C	GLA	27	-11.442	-0.266	13.259	1.00	0.00
ATOM	284	O	GLA	27	-11.085	0.123	14.373	1.00	0.00
ATOM	285	CB	GLA	27	-12.305	-2.670	13.129	1.00	0.00
ATOM	286	CG	GLA	27	-13.477	-3.637	13.409	1.00	0.00
ATOM	287	CD	GLA	27	-13.027	-5.030	13.859	1.00	0.00
ATOM	288	OE2	GLA	27	-13.677	-5.580	14.778	1.00	0.00
ATOM	289	OE1	GLA	27	-12.065	-5.577	13.274	1.00	0.00
ATOM	290	C12	GLA	27	-14.419	-3.750	12.195	1.00	0.00
ATOM	291	O13	GLA	27	-13.927	-4.095	11.089	1.00	0.00
ATOM	292	O14	GLA	27	-15.653	-3.726	12.417	1.00	0.00
ATOM	293	N	ALA	28	-10.776	0.092	12.157	1.00	0.00
ATOM	294	CA	ALA	28	-9.564	0.896	12.186	1.00	0.00
ATOM	295	C	ALA	28	-9.806	2.383	12.481	1.00	0.00
ATOM	296	O	ALA	28	-8.865	3.094	12.844	1.00	0.00
ATOM	297	CB	ALA	28	-8.772	0.680	10.898	1.00	0.00
ATOM	298	H	ALA	28	-11.171	-0.143	11.254	1.00	0.00
ATOM	299	N	ARG	29	-11.041	2.872	12.330	1.00	0.00
ATOM	300	CA	ARG	29	-11.359	4.241	12.697	1.00	0.00
ATOM	301	C	ARG	29	-11.349	4.382	14.216	1.00	0.00
ATOM	302	O	ARG	29	-10.652	5.246	14.749	1.00	0.00
ATOM	303	CB	ARG	29	-12.696	4.673	12.090	1.00	0.00
ATOM	304	CG	ARG	29	-12.749	6.192	11.915	1.00	0.00
ATOM	305	CD	ARG	29	-13.939	6.778	12.678	1.00	0.00
ATOM	306	NE	ARG	29	-13.788	6.575	14.123	1.00	0.00
ATOM	307	CZ	ARG	29	-12.827	7.203	14.812	1.00	0.00
ATOM	308	NH1	ARG	29	-12.093	8.104	14.156	1.00	0.00
ATOM	309	NH2	ARG	29	-12.596	6.958	16.103	1.00	0.00
ATOM	310	H	ARG	29	-11.810	2.246	12.100	1.00	0.00
ATOM	311	HE	ARG	29	-14.366	5.848	14.567	1.00	0.00
ATOM	312	HH21	ARG	29	-13.193	6.275	16.588	1.00	0.00
ATOM	313	HH22	ARG	29	-11.737	7.251	16.540	1.00	0.00
ATOM	314	HH11	ARG	29	-12.244	8.188	13.160	1.00	0.00
ATOM	315	HH12	ARG	29	-11.294	8.569	14.580	1.00	0.00
ATOM	316	N	GLA	30	-12.122	3.537	14.907	1.00	0.00
ATOM	317	CA	GLA	30	-12.218	3.504	16.360	1.00	0.00
ATOM	318	C	GLA	30	-10.838	3.335	17.007	1.00	0.00
ATOM	319	O	GLA	30	-10.510	4.062	17.945	1.00	0.00
ATOM	320	CB	GLA	30	-13.253	2.460	16.826	1.00	0.00
ATOM	321	CG	GLA	30	-14.724	2.951	16.851	1.00	0.00
ATOM	322	CD	GLA	30	-14.856	4.429	16.438	1.00	0.00
ATOM	323	OE2	GLA	30	-15.359	4.703	15.325	1.00	0.00
ATOM	324	OE1	GLA	30	-14.387	5.334	17.174	1.00	0.00
ATOM	325	C12	GLA	30	-15.436	2.701	18.189	1.00	0.00
ATOM	326	O13	GLA	30	-16.668	2.926	18.191	1.00	0.00
ATOM	327	O14	GLA	30	-14.742	2.772	19.231	1.00	0.00
ATOM	328	N	VAL	31	-10.013	2.416	16.486	1.00	0.00
ATOM	329	CA	VAL	31	-8.689	2.124	17.030	1.00	0.00
ATOM	330	C	VAL	31	-7.759	3.335	17.164	1.00	0.00
ATOM	331	O	VAL	31	-7.436	3.735	18.282	1.00	0.00
ATOM	332	CB	VAL	31	-8.033	0.926	16.334	1.00	0.00
ATOM	333	CG1	VAL	31	-7.934	1.110	14.824	1.00	0.00
ATOM	334	CG2	VAL	31	-6.629	0.691	16.884	1.00	0.00
ATOM	335	H	VAL	31	-10.369	1.821	15.744	1.00	0.00
ATOM	336	N	PHE	32	-7.266	3.682	16.047	1.00	0.00
ATOM	337	CA	PHE	32	-6.219	4.894	16.127	1.00	0.00
ATOM	338	C	PHE	32	-6.627	6.276	15.615	1.00	0.00
ATOM	339	O	PHE	32	-5.957	6.846	14.746	1.00	0.00
ATOM	340	CB	PHE	32	-4.889	4.388	15.562	1.00	0.00
ATOM	341	CG	PHE	32	-3.677	4.777	16.387	1.00	0.00
ATOM	342	CD1	PHE	32	-3.628	4.461	17.757	1.00	0.00
ATOM	343	CD2	PHE	32	-2.601	5.453	15.787	1.00	0.00
ATOM	344	CE1	PHE	32	-2.507	4.827	18.527	1.00	0.00
ATOM	345	CE2	PHE	32	-1.480	5.820	16.557	1.00	0.00
ATOM	346	CZ	PHE	32	-1.434	5.506	17.926	1.00	0.00
ATOM	347	H	PHE	32	-7.575	3.551	15.142	1.00	0.00
ATOM	348	N	GLA	33	-7.676	6.852	16.210	1.00	0.00
ATOM	349	CA	GLA	33	-8.010	8.270	16.148	1.00	0.00
ATOM	350	C	GLA	33	-8.625	8.732	14.826	1.00	0.00

ATOM	351	O	GLA	33	-9.769	9.184	14.811	1.00	0.00
ATOM	352	CB	GLA	33	-6.800	9.130	16.497	1.00	0.00
ATOM	353	CG	GLA	33	-6.696	9.571	17.962	1.00	0.00
ATOM	354	CD	GLA	33	-7.894	10.411	18.437	1.00	0.00
ATOM	355	OE2	GLA	33	-7.990	10.683	19.657	1.00	0.00
ATOM	356	OE1	GLA	33	-8.733	10.774	17.581	1.00	0.00
ATOM	357	C12	GLA	33	-5.347	10.254	18.208	1.00	0.00
ATOM	358	O13	GLA	33	-4.829	10.809	17.205	1.00	0.00
ATOM	359	O14	GLA	33	-4.664	9.805	19.159	1.00	0.00
ATOM	360	N	ASN	34	-7.879	8.697	13.720	1.00	0.00
ATOM	361	CA	ASN	34	-8.290	9.433	12.532	1.00	0.00
ATOM	362	C	ASN	34	-8.120	8.690	11.211	1.00	0.00
ATOM	363	O	ASN	34	-7.011	8.309	10.841	1.00	0.00
ATOM	364	CB	ASN	34	-7.762	10.874	12.535	1.00	0.00
ATOM	365	CG	ASN	34	-6.464	11.076	11.759	1.00	0.00
ATOM	366	OD1	ASN	34	-5.372	11.001	12.320	1.00	0.00
ATOM	367	ND2	ASN	34	-6.563	11.385	10.472	1.00	0.00
ATOM	368	H	ASN	34	-6.982	8.226	13.746	1.00	0.00
ATOM	369	HD21	ASN	34	-7.480	11.502	10.017	1.00	0.00
ATOM	370	HD22	ASN	34	-5.728	11.509	9.925	1.00	0.00
ATOM	371	N	THR	35	-9.244	8.524	10.511	1.00	0.00
ATOM	372	CA	THR	35	-9.430	7.841	9.238	1.00	0.00
ATOM	373	C	THR	35	-8.316	8.113	8.226	1.00	0.00
ATOM	374	O	THR	35	-7.825	7.197	7.562	1.00	0.00
ATOM	375	CB	THR	35	-10.781	8.296	8.677	1.00	0.00
ATOM	376	OG1	THR	35	-11.675	8.608	9.734	1.00	0.00
ATOM	377	CG2	THR	35	-11.[illegible]	7.236	7.766	1.00	0.00
ATOM	378	H	THR	35	-10.109	8.895	10.884	1.00	0.00
ATOM	379	HG1	THR	35	-12.563	8.373	9.455	1.00	0.00
ATOM	380	N	GLU	36	-7.928	9.380	8.099	1.00	0.00
ATOM	381	CA	GLU	36	-6.874	9.836	7.216	1.00	0.00
ATOM	382	C	GLU	36	-5.564	9.079	7.459	1.00	0.00
ATOM	383	O	GLU	36	-4.876	8.726	6.504	1.00	0.00
ATOM	384	CB	GLU	36	-6.711	11.347	7.395	1.00	0.00
ATOM	385	CG	GLU	36	-7.971	12.106	6.950	1.00	0.00
ATOM	386	CD	GLU	36	-9.059	12.252	8.016	1.00	0.00
ATOM	387	OE1	GLU	36	-8.878	11.681	9.119	1.00	0.00
ATOM	388	OE2	GLU	36	-10.077	12.901	7.694	1.00	0.00
ATOM	389	H	GLU	36	-8.395	10.098	8.653	1.00	0.00
ATOM	390	N	ARG	37	-5.237	8.818	8.729	1.00	0.00
ATOM	391	CA	ARG	37	-4.113	7.974	9.106	1.00	0.00
ATOM	392	C	ARG	37	-4.525	6.505	9.007	1.00	0.00
ATOM	393	O	ARG	37	-3.865	5.708	8.340	1.00	0.00
ATOM	394	CB	ARG	37	-3.716	8.284	10.546	1.00	0.00
ATOM	395	CG	ARG	37	-2.522	9.229	10.620	1.00	0.00
ATOM	396	CD	ARG	37	-2.006	9.244	12.057	1.00	0.00
ATOM	397	NE	ARG	37	-3.072	9.609	12.993	1.00	0.00
ATOM	398	CZ	ARG	37	-3.761	8.722	13.721	1.00	0.00
ATOM	399	NH1	ARG	37	-3.589	7.414	13.518	1.00	0.00
ATOM	400	NH2	ARG	37	-4.625	9.128	14.651	1.00	0.00
ATOM	401	H	ARG	37	-5.897	9.064	9.459	1.00	0.00
ATOM	402	HE	ARG	37	-3.480	10.532	12.895	1.00	0.00
ATOM	403	HH21	ARG	37	-4.682	10.077	15.010	1.00	0.00
ATOM	404	HH22	ARG	37	-5.176	8.410	15.121	1.00	0.00
ATOM	405	HH11	ARG	37	-2.907	7.082	12.848	1.00	0.00
ATOM	406	HH12	ARG	37	-4.179	6.755	14.023	1.00	0.00
ATOM	407	N	THR	38	-5.634	6.165	9.673	1.00	0.00
ATOM	408	CA	THR	38	-6.247	4.848	9.722	1.00	0.00
ATOM	409	C	THR	38	-6.116	4.081	8.408	1.00	0.00
ATOM	410	O	THR	38	-5.618	2.961	8.398	1.00	0.00
ATOM	411	CB	THR	38	-7.702	5.003	10.166	1.00	0.00
ATOM	412	OG1	THR	38	-7.758	5.325	11.538	1.00	0.00
ATOM	413	CG2	THR	38	-8.530	3.752	9.895	1.00	0.00
ATOM	414	H	THR	38	-6.101	6.883	10.213	1.00	0.00
ATOM	415	HG1	THR	38	-8.087	4.551	12.020	1.00	0.00
ATOM	416	N	THR	39	-6.528	4.685	7.293	1.00	0.00
ATOM	417	CA	THR	39	-6.468	4.053	5.983	1.00	0.00
ATOM	418	C	THR	39	-5.055	3.634	5.566	1.00	0.00
ATOM	419	O	THR	39	-4.893	2.586	4.934	1.00	0.00
ATOM	420	CB	THR	39	-7.175	4.904	4.924	1.00	0.00
ATOM	421	OG1	THR	39	-6.886	6.278	5.075	1.00	0.00
ATOM	422	CG2	THR	39	-8.684	4.715	5.039	1.00	0.00
ATOM	423	H	THR	39	-6.909	5.620	7.365	1.00	0.00

ATOM	424	HG1	THR	39	-7.357	6.609	5.852	1.00	0.00
ATOM	425	N	GLU	40	-4.044	4.439	5.911	1.00	0.00
ATOM	426	CA	GLU	40	-2.640	4.135	5.680	1.00	0.00
ATOM	427	C	GLU	40	-2.185	2.968	6.557	1.00	0.00
ATOM	428	O	GLU	40	-1.577	2.019	6.062	1.00	0.00
ATOM	429	CB	GLU	40	-1.770	5.382	5.885	1.00	0.00
ATOM	430	CG	GLU	40	-2.355	6.642	5.236	1.00	0.00
ATOM	431	CD	GLU	40	-1.355	7.378	4.344	1.00	0.00
ATOM	432	OE1	GLU	40	-0.636	8.259	4.860	1.00	0.00
ATOM	433	OE2	GLU	40	-1.360	7.037	3.137	1.00	0.00
ATOM	434	H	GLU	40	-4.234	5.230	6.520	1.00	0.00
ATOM	435	N	PHE	41	-2.519	3.016	7.850	1.00	0.00
ATOM	436	CA	PHE	41	-2.310	1.888	8.752	1.00	0.00
ATOM	437	C	PHE	41	-2.931	0.612	8.171	1.00	0.00
ATOM	438	O	PHE	41	-2.304	-0.445	8.140	1.00	0.00
ATOM	439	CB	PHE	41	-2.903	2.208	10.124	1.00	0.00
ATOM	440	CG	PHE	41	-2.081	3.159	10.963	1.00	0.00
ATOM	441	CD1	PHE	41	-1.131	2.655	11.870	1.00	0.00
ATOM	442	CD2	PHE	41	-2.321	4.544	10.897	1.00	0.00
ATOM	443	CE1	PHE	41	-0.417	3.537	12.701	1.00	0.00
ATOM	444	CE2	PHE	41	-1.597	5.426	11.717	1.00	0.00
ATOM	445	CZ	PHE	41	-0.647	4.923	12.623	1.00	0.00
ATOM	446	H	PHE	41	-2.995	3.845	8.201	1.00	0.00
ATOM	447	N	TRP	42	-4.160	0.739	7.673	1.00	0.00
ATOM	448	CA	TRP	42	-4.919	-0.266	6.956	1.00	0.00
ATOM	449	C	TRP	42	-4.134	-0.795	5.755	1.00	0.00
ATOM	450	O	TRP	42	-4.042	-2.006	5.565	1.00	0.00
ATOM	451	CB	TRP	42	-6.230	0.390	6.532	1.00	0.00
ATOM	452	CG	TRP	42	-7.285	-0.454	5.895	1.00	0.00
ATOM	453	CD1	TRP	42	-8.311	0.049	5.178	1.00	0.00
ATOM	454	CD2	TRP	42	-7.484	-1.901	5.946	1.00	0.00
ATOM	455	NE1	TRP	42	-9.094	-0.995	4.732	1.00	0.00
ATOM	456	CE2	TRP	42	-8.636	-2.216	5.170	1.00	0.00
ATOM	457	CE3	TRP	42	-6.820	-2.981	6.569	1.00	0.00
ATOM	458	CZ2	TRP	42	-9.100	-3.530	5.001	1.00	0.00
ATOM	459	CZ3	TRP	42	-7.290	-4.299	6.430	1.00	0.00
ATOM	460	CH2	TRP	42	-8.415	-4.579	5.632	1.00	0.00
ATOM	461	H	TRP	42	-4.603	1.640	7.791	1.00	0.00
ATOM	462	HE1	TRP	42	-9.938	-0.838	4.197	1.00	0.00
ATOM	463	N	LYS	43	-3.551	0.103	4.950	1.00	0.00
ATOM	464	CA	LYS	43	-2.611	-0.284	3.904	1.00	0.00
ATOM	465	C	LYS	43	-1.527	-1.197	4.479	1.00	0.00
ATOM	466	O	LYS	43	-1.429	-2.359	4.087	1.00	0.00
ATOM	467	CB	LYS	43	-1.975	0.952	3.273	1.00	0.00
ATOM	468	CG	LYS	43	-3.030	1.856	2.652	1.00	0.00
ATOM	469	CD	LYS	43	-2.662	3.303	2.953	1.00	0.00
ATOM	470	CE	LYS	43	-3.699	4.247	2.362	1.00	0.00
ATOM	471	NZ	LYS	43	-3.509	5.607	2.873	1.00	0.00
ATOM	472	H	LYS	43	-3.668	1.093	5.149	1.00	0.00
ATOM	473	HZ3	LYS	43	-2.539	5.913	2.775	1.00	0.00
ATOM	474	HZ2	LYS	43	-3.699	5.642	3.871	1.00	0.00
ATOM	475	HZ1	LYS	43	-4.097	6.274	2.392	1.00	0.00
ATOM	476	N	GLN	44	-0.757	-0.673	5.442	1.00	0.00
ATOM	477	CA	GLN	44	0.254	-1.411	6.192	1.00	0.00
ATOM	478	C	GLN	44	-0.226	-2.823	6.531	1.00	0.00
ATOM	479	O	GLN	44	0.468	-3.806	6.286	1.00	0.00
ATOM	480	CB	GLN	44	0.544	-0.656	7.487	1.00	0.00
ATOM	481	CG	GLN	44	1.801	0.211	7.426	1.00	0.00
ATOM	482	CD	GLN	44	1.919	1.117	8.640	1.00	0.00
ATOM	483	OE1	GLN	44	2.706	2.056	8.648	1.00	0.00
ATOM	484	NE2	GLN	44	1.135	0.866	9.684	1.00	0.00
ATOM	485	H	GLN	44	-0.936	0.292	5.705	1.00	0.00
ATOM	486	HE21	GLN	44	0.423	0.148	9.629	1.00	0.00
ATOM	487	HE22	GLN	44	1.119	1.554	10.429	1.00	0.00
ATOM	488	N	TYR	45	-1.428	-2.897	7.099	1.00	0.00
ATOM	489	CA	TYR	45	-2.111	-4.124	7.445	1.00	0.00
ATOM	490	C	TYR	45	-2.309	-5.041	6.233	1.00	0.00
ATOM	491	O	TYR	45	-1.640	-6.065	6.109	1.00	0.00
ATOM	492	CB	TYR	45	-3.446	-3.753	8.091	1.00	0.00
ATOM	493	CG	TYR	45	-4.056	-4.843	8.930	1.00	0.00
ATOM	494	CD1	TYR	45	-3.975	-4.773	10.330	1.00	0.00
ATOM	495	CD2	TYR	45	-4.631	-5.966	8.310	1.00	0.00
ATOM	496	CE1	TYR	45	-4.446	-5.839	11.112	1.00	0.00

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ATOM    497  CE2 TYR    45      -5.075  -7.044   9.092  1.00  0.00
ATOM    498  CZ  TYR    45      -4.980  -6.980  10.491  1.00  0.00
ATOM    499  OH  TYR    45      -5.305  -8.071  11.232  1.00  0.00
ATOM    500  H   TYR    45      -1.912  -2.027   7.281  1.00  0.00
ATOM    501  HH  TYR    45      -5.445  -7.857  12.167  1.00  0.00
ATOM    502  N   VAL    46      -3.268  -4.708   5.369  1.00  0.00
ATOM    503  CA  VAL    46      -3.804  -5.684   4.436  1.00  0.00
ATOM    504  C   VAL    46      -3.153  -5.684   3.053  1.00  0.00
ATOM    505  O   VAL    46      -3.278  -6.680   2.340  1.00  0.00
ATOM    506  CB  VAL    46      -5.330  -5.588   4.377  1.00  0.00
ATOM    507  CG1 VAL    46      -5.798  -4.499   3.411  1.00  0.00
ATOM    508  CG2 VAL    46      -5.945  -6.933   4.002  1.00  0.00
ATOM    509  H   VAL    46      -3.745  -3.818   5.484  1.00  0.00
ATOM    510  N   ASP    47      -2.488  -4.587   2.661  1.00  0.00
ATOM    511  CA  ASP    47      -1.986  -4.347   1.307  1.00  0.00
ATOM    512  C   ASP    47      -1.397  -5.608   0.677  1.00  0.00
ATOM    513  O   ASP    47      -1.870  -6.090  -0.351  1.00  0.00
ATOM    514  CB  ASP    47      -0.958  -3.218   1.355  1.00  0.00
ATOM    515  CG  ASP    47      -1.085  -2.236   0.201  1.00  0.00
ATOM    516  OD1 ASP    47      -2.174  -1.639   0.055  1.00  0.00
ATOM    517  OD2 ASP    47      -0.062  -2.076  -0.501  1.00  0.00
ATOM    518  H   ASP    47      -2.348  -3.836   3.330  1.00  0.00
ATOM    519  N   GLY    48      -0.408  -6.181   1.362  1.00  0.00
ATOM    520  CA  GLY    48       0.081  -7.512   1.064  1.00  0.00
ATOM    521  C   GLY    48       0.053  -8.373   2.325  1.00  0.00
ATOM    522  O   GLY    48       1.087  -8.923   2.697  1.00  0.00
ATOM    523  OXT GLY    48      -1.113  -8.465   2.968  1.00  0.00
ATOM    524  H   GLY    48      -0.082  -5.708   2.194  1.00  0.00
TER     525      GLY    48
ATOM    526  C0  UNN          -17.483   2.887  14.115  1.00  0.00
TER     527      UNN
ATOM    528  C0  UNN          -16.224  -1.256  15.982  1.00  0.00
TER     529      UNN
ATOM    530  C0  UNN          -12.372  -1.241  17.496  1.00  0.00
TER     531      UNN
ATOM    532  C0  UNN          -12.763  -4.581  17.359  1.00  0.00
TER     533      UNN
ATOM    534  C0  UNN          -10.810  -8.201  18.729  1.00  0.00
TER     535      UNN
ATOM    536  C0  UNN           -6.910 -11.401  20.105  1.00  0.00
TER     537      UNN
ATOM    538  C0  UNN            1.480  -8.643  19.524  1.00  0.00
TER     539      UNN
CONECT  188  187  190  191  245
CONECT  245  188  244  247  248
MASTER        0    0    0    0    0    0    0    0  531    8    2   11
END
```

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Temple University - Of The Commonwealth System of Higher Education

(ii) INVENTORS: Walsh, Peter N., Ahmad, Syed S. and Jameson, Bradford A.

(iii) TITLE OF INVENTION: PEPTIDE ANALOGS OF THE FACTOR IXa PLATELET BINDING SITE

(iv) NUMBER OF SEQUENCES: 4

(v) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Seidel, Gonda, Lavorgna & Monaco, P.C.

(B) STREET: Two Penn Center Plaza, Suite 1800

(C) CITY: Philadelphia

(D) STATE: Pennsylvania

(E) COUNTRY: U.S.A.

(F) ZIP: 19103

(vi) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.50 inch, 720 Kb

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: WordPerfect 5.1

(vii) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) PRIORIRY APPLICATION DATA:

(A) APPLICATION NUMBER: 08/172,330

(B) FILING DATE: 12/22/93

(ix) ATTORNEY/AGENT INFORMATION:

(A) NAME: Monaco, Daniel A.

(B) REGISTRATION NUMBER: 30,480

(C) REFERENCE/DOCKET NUMBER: 6056-187PC

(x) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 568-8383

(B) TELEFAX: (215) 568-5549

(2) **INFORMATION FOR SEQ ID NO:1:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 48 amino acids

(B) **TYPE:** amino acid

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:1:**

Tyr Asn Ser Gly Lys Leu Xaa Xaa Phe Val Gln Gly Asn Leu Xaa
5 10 15

Arg Xaa Cys Met Xaa Xaa Lys Cys Ser Phe Xaa Xaa Ala Arg Xaa
20 25 30

Val Phe Xaa Asn Thr Glu Arg Thr Thr Glu Phe Trp Lys Gln Tyr
35 40 45

Val Asp Gln
48

(3) **INFORMATION FOR SEQ ID NO:2:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 12 amino acids

(B) **TYPE:** amino acid

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:2:**

Cys Pro Gly Lys Leu Asp Glu Phe Val Gln Pro Cys
5 10

(4) **INFORMATION FOR SEQ ID NO:3:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 6 amino acids

(B) **TYPE:** amino acid

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:3:**

Cys Ser Gly Lys Leu Cys
5

(5) **INFORMATION FOR SEQ ID NO:4:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 5 amino acids

(B) **TYPE:** amino acid

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:4:**

Cys Phe Val Gln Cys
5

Claims

1. A synthetic peptide, or a pharmaceutically acceptable salt thereof, said peptide consisting essentially of an amino acid sequence from at least 5 to about 75 amino acids in length, which sequence corresponds to a portion of the amino acid sequence of the platelet binding site on factor IXa, said peptide having an artificially introduced restricted conformation free of adjacent γ-carboxyglutamic acid residues and the ability to inhibit the binding of factor IXa to a platelet surface.

2. A peptide according to claim 1 wherein the peptide is free of γ-carboxyglutamic acid residues.

3. A peptide according to claim 2 wherein the peptide is from 5 to about 45 amino acids in length.

4. A peptide according to claim 3 wherein the peptide is from about 5 to about 20 amino acids in length.

5. A peptide according to claim 2 wherein the conformation is restricted by means of at least one cysteine-cysteine disulfide bond.

6. A peptide according to claim 2 wherein the restricted conformation is determined from the equilibrium conformation model comprising the set of coordinates and connect statements of Appendix 1.

7. A synthetic peptide, or a pharmaceutically acceptable salt thereof, said peptide consisting essentially of an amino acid sequence from at least 5 to about 75 amino acids in length, which amino acid sequence corresponds to a portion of the amino acid sequence of the platelet binding site on factor IXa,

wherein said restricted conformation is provided at least in part by:

(i) at least one cysteine-cysteine disulfide bond which is not present in the native amino acid sequence of factor XIa, or

(ii) at least one artificially introduced covalent bond other than a disulfide bond.

8. A peptide according to claim 7 wherein the peptide is from about 5 to about 45 amino acids in length.

9. A peptide according to claim 8 wherein the peptide is from about 5 to about 20 amino acids in length.

10. A peptide according to claim 7, wherein the conformation is restricted by means of at least one cysteine-cysteine disulfide bond.

11. A peptide according to claim 7 wherein the restricted conformation is determined from the equilibrium conformation model comprising the set of coordinates and connect statements of Appendix 1.

12. A peptide according to claim 7 wherein the conformation is restricted at least in part by at least one amide bond.

13. A peptide according to claim 7 wherein the conformation is restricted at least in part by at least one toluene-2,4-diisocyanate cross-link between two free amino groups of the peptide.

14. A peptide according to claim 13 wherein the conformation is restricted at least in part by at least one amide bond formed between side chains of a lysine residue and a glutamic or aspartic acid residue of the peptide.

15. A peptide according to claim 7 wherein the amino acid sequence of said peptide comprises amino acids 4-6 or 9-11 of SEQ ID NO:1, or combinations thereof.

16. A peptide according to claim 15 having an amino acid sequence selected from the group of sequences consisting of:

SEQ ID NO:2;

SEQ ID NO:3;

SEQ ID NO:4; and

combinations thereof.

17. A peptide according to claim 16 having an amino acid sequence of SEQ ID NO:2.

18. A method of designing a synthetic peptide or a pharmaceutically acceptable salt thereof, said peptide consisting essentially of an amino acid sequence from at least 5 to about 75 amino acids in length, which sequence corresponds to a portion of the amino acid sequence of the platelet binding site on factor IXa, said peptide having an artificially introduced restricted conformation and the ability to inhibit the binding of factor IXa to a platelet surface, comprising:

determining the distance between two parts of a molecular model including the factor IXa platelet binding site at conformational equilibrium;

more cysteine residues to form an intramolecular disulfide bond, or forming an amide bond linking two parts of the primary structure of the platelet binding site.

22. The method according to claim 21 wherein the step of modifying the primary structure comprises introducing an amino acid selected from the group consisting of lysine, glutamic acid and aspartic acid and reacting side chains of a lysine with a glutamic or aspartic acid residue to form an amide bond internally cross-linking two parts of the platelet binding site.

23. The method according to claim 18 wherein the step of modifying the primary structure comprises introducing a toluene-2,4-diisocyanate structure to internally cross-link two free amino groups of the peptide.

24. The method according to claim 18 wherein the molecular model comprises the set of coordinates and connect statements of Appendix 1.

25. A method of producing a synthetic peptide, or a pharmaceutically acceptable salt thereof, said peptide consisting essentially of an amino acid sequence from at least 5 to about 75 amino acids in length, which sequence corresponds to a portion of the amino acid sequence of the platelet binding site on factor IXa, said peptide having an artificially introduced restricted conformation and the ability to inhibit the binding of factor IXa to a platelet surface, comprising:

providing a peptide having an amino acid sequence corresponding to a portion of the sequence of the platelet binding site on the factor IXa chain;

determining the conformational equilibrium of that portion of the factor IXa chain; and

introducing a covalent modification into the peptide to restrict a distance determined to be between two parts of the peptide to a distance between two corresponding parts of the peptide in the equilibrium conformation.

26. A method according to claim 25 whereinintroducing a covalent modification comprises restricting a distance determined to be between two parts of the peptide to a distance between two corresponding parts of the peptide in the equilibrium conformation by introducing a cysteine residue not present in the native amino acid sequence to form a cysteine-cysteine disulfide bond with another cysteine residue or by introducing a covalent bond other than a cysteine-cysteine disulfide bond.

27. A method according to claim 26 wherein said covalent modification comprises an amide bond cross-linking two parts of the peptide.

28. A method according to claim 26 wherein said synthetic peptide is free of γ-carboxyglutamic acid residues.

29. A pharmaceutical composition comprising one or more peptides of claim 1, or a pharmaceutically acceptable salt of said peptide, and a pharmaceutically acceptable carrier.

30. A pharmaceutical composition comprising a peptide of claim 2, or a pharmaceutically acceptable salt of said peptide, and a pharmaceutically acceptable carrier.

31. A pharmaceutical composition comprising a peptide of claim 7, or a pharmaceutically acceptable salt of said peptide, and a pharmaceutically acceptable carrier.

32. A method of inhibiting factor IXa-induced activation of factor X on the platelet surface comprising contacting a platelet with one or more synthetic peptides according to claim 1, or a pharmaceutically acceptable salt thereof.

33. A method according to claim 32 wherein an amino acid sequence segment of said peptide is selected from the group of consisting of:

SEQ ID NO:2;
SEQ ID NO:3;
SEQ ID NO:4; and
combinations thereof.

34. A method according to claim 32 wherein said peptide is free of γ-carboxyglutamic acid residues.

35. A method of inhibiting the binding of factor IXa to a platelet surface comprising contacting a platelet surface with one or more synthetic peptides comprising an amino acid sequence corresponding to a portion of the sequence of the platelet binding site on the factor IXa chain, said peptide having an artificially introduced restricted conformation and the ability to inhibit the binding of factor IXa to a platelet surface.

36. A method according to claim 35 wherein said peptide is free of γ-carboxyglutamic acid residues.

37. A method according to claim 36 wherein said peptide comprises an amino acid sequence selected from the group consisting of:
SEQ ID NO:2;
SEQ ID NO:3;
SEQ ID NO:4; and
combinations thereof.

38. A method for inhibiting thrombosis comprising administering to a mammal in need of such treatment an effective amount of one or more synthetic peptides according to claim 1, or a pharmaceutically acceptable salt of said peptide.

39. A method for inhibiting thrombosis comprising administering to a mammal in need of such treatment an effective amount of one or more synthetic peptides according to claim 2, or a pharmaceutically acceptable salt of said peptide.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14016

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 7/00, 7/06, 7/08, 14/00; A61K 38/08, 38/10, 38/16
US CL :530/330, 329, 328, 327, 326, 325, 324; 514/12, 13, 14, 15, 16, 17
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/330, 329, 328, 327, 326, 325, 324; 514/12, 13, 14, 15, 16, 17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Blood, Volume 79, No. 2, issued 15 January 1992, R. Rawala-Sheikh, "Role of γ-Carboxyglutamic Acid Residues in the Binding of Factor IXa to Platelets and in Factor-X Activation", pages 398-405, see entire document.	1-39
A	Biochemistry, Volume 25, issued 1986, A. D. Turner, "p-Amidino Esters as Irreversible Inhibitors of Factors IXa and Xa and Thrombin", pages 4929-4935, see entire document.	1-39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date f the actual completion of the international search
21 MARCH 1995

Date of mailing of the international search report
29 MAR 1995

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14016

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	The Journal of Biological Chemistry, Volume 267, No. 5, issued 15 February 1992, J. Astermark, "Effects of γ-Carboxyglutamic Acid and Epidermal Growth Factor-like Modules of Factor IX on Factor X Activation", pages 3249-3256, see entire document.	1-39
A	The Journal of Biological Chemistry, Volume 267, No. 12, issued 25 April 1992, S. S. Ahmad, " The Role of the First Growth Factor Domain of Human Factor IXa in Binding to Platelets and in Factor X Activation", pages 8571-8576, see entire document.	1-39
A	The Journal of Biological Chemistry, Volume 266, No. 35, issued 15 December 1991, F. A. Baglia, "Identification and Chemical Synthesis of a Substrate-binding Site for Factor IX on Coagulation Factor XIa", pages 24190-24197, see entire document.	1-39